CHARACTERIZATION OF SYSTEMIC ACQUIRED RESISTANCE IN *BRASSICA NAPUS*

A Thesis Submitted to the College of

Graduate Studies and Research

In Partial Fulfillment of the Requirements

For the Degree of Doctor of Philosophy

In the Department of Biology

University of Saskatchewan

Saskatoon, Canada

By

Shobha Devi Potlakayala

PERMISSION TO USE

In presenting this thesis in partial fulfilment of the requirements for a Postgraduate degree from the University of Saskatchewan, I agree that the Libraries of this University may make it freely available for inspection. I further agree that permission for copying of this thesis in any manner, in whole or in part, for scholarly purposes may be granted by the professor or professors who supervised my thesis work or, in their absence, by the Head of the Department or the Dean of the College in which my thesis work was done. It is understood that any copying or publication or use of this thesis or parts thereof for financial gain shall not be allowed without my written permission. It is also understood that due recognition shall be given to me and to the University of Saskatchewan in any scholarly use which may be made of any material in my thesis.

Requests for permission to copy or to make other use of material in this thesis in whole or part should be addressed to:

Head of the Department of Biology

University of Saskatchewan

Saskatoon, Saskatchewan, Canada S7N 5E2

ABSTRACT

Plants activate an array of defense mechanisms upon pathogen attack. Systemic acquired resistance (SAR) is an induced disease resistance phenomenon deployed after infection by a necrogenic pathogen and is dependent on endogenous accumulation of salicylic acid. The objectives of my research were to characterize SAR in the crop plant, *Brassica napus* (canola), and study the effects of overexpressing genes involved in SAR on disease resistance. Biological induction of SAR using necrogenic *Pseudomonas syringae* and chemical induction using benzo (1,2,3) thiadiazole-7-carbothionic acid reduced growth of the bacterial pathogen *P. syringae* and the fungal pathogen *Leptosphaeria maculans*. This growth reduction was associated with an increase in transcript levels of pathogenesis-related (*PR*) genes, one of the characteristic features of SAR. Transgenic plants expressing a bacterial salicylate hydroxylase gene (*NahG*), were more susceptible to the above pathogens and were delayed in accumulating *PR* gene transcripts, indicating a need for SA accumulation for SAR in *B. napus*. Expression of two SAR genes from *Arabidopsis*, *DEFECTIVE IN INDUCED RESISTANCE 1* (*DIR1*) and *NON EXPRESSOR OF PATHOGENESIS-RELATED 1* (*NPR1*), in *B. napus* enhanced resistance against virulent *P. syringae* without SAR pre-treatments. Putative orthologs of *DIR1* and *NPR1* (*BnDIR1* and *BnNPR1*) were isolated from *B. napus* based on EST sequences. BnDIR1 and BnNPR1 display 71% and 66% amino acid sequence similarities, respectively, to the corresponding *Arabidopsis* proteins. Expression of *BnNPR1* in *Arabidopsis npr1* mutant backgrounds indicated that it was able to functionally complement these mutations. Expression of *BnDIR1* enhanced disease resistance in both *Arabidopsis* wild-type and *dir1-1* mutant backgrounds. Expression of *DIR1*, *NPR1, BnDIR1* and *BnNPR1*, separately, in *B. napus* plants enhanced resistance against *P. syringae*. SAR pre-treatments further enhanced resistance of transgenic *B. napus* plants expressing *DIR1* and *BnDIR1* to *P. syringae*, indicating an additive effect. Expression of *DIR1* in *B. napus* did not provide resistance against *L. maculans*. These results provide the first in-depth molecular characterization of SAR in *B. napus*, and in particular, provide new insight into DIR1 function not previously reported in *Arabidopsis*.

ACKNOWLEDGEMENTS

I would like to thank my supervisor, Dr. Pierre Fobert for his enthusiastic guidance and support throughout my PhD program. I will always be very grateful for giving me the chance to work in his lab and I greatly appreciate all his patience, especially during thesis writing.

I would also like to thank my committee members, Dr. Peta Bonham-Smith, Dr. Louise Nelson, Dr. Roger Rimmer and Dr. Yangdou Wei and Head of the Department Dr. François Messier (and former Heads Dr. Larry Fowke and Dr. Vipen Sawhney) for all their input and help. I would like to thank Dr. Robin Cameron for serving as my external examiner.

I would also like to thank all the scientists and staff from Agriculture and Agri-Food Canada, Department of Biology and Plant Biotechnology Institute who helped me with my research. I would like to thank all the individuals who provided me with pathogen strains and other research materials for my project. I would like to express my gratitude to the University of Saskatchewan and Natural Sciences and Engineering Research Council of Canada for their financial support.

I would like to thank all my friends and especially Catherine DeLong and Kerri McIntosh for their support and friendship. Lastly, and most importantly, I would like to thank my parents, all my sisters and my brother for their love and constant support. A great big thank you to Dr. Sairam Rudrabhatla, the love of my life, for all the care, support, love and endless patience.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

aa	amino acid
°C	degrees centigrade
%	percentage
µ	micro-
α-^{32}P dCTP	radiolabelled 2'-deoxycytidine 5'-triphosphate
AAFC	Agriculture and Agrifood Canada, Saskatoon Research Station, Canada
A. brassicicola	*Alternaria brassicicola*
ABRC	*Arabidopsis* Biological Resources Center
ACC	1-aminocyclopropane-1-carboxylate
a.i.	active ingredient
ARD	ankyrin repeat domain
as-1	activating sequence 1
AtDIR1	*Arabidopsis DIR 1*
AGI	*Arabidopsis* Genome Initiative
A. thaliana	*Arabidopsis thaliana*
ATP	adenosine triphosphate
A. tumefaciens	*Agrobacterium tumefaciens*
Avr-gene	*Avirulence* gene
BABA	β-aminobutyric acid
BABA-IR	BABA-induced resistance
BBRC	Biotechnology and Biological Sciences Research Council
BC	Before Christ
B. carinata	*Brassica carinata*
B. cinerea	*Botrytis cinerea*
BLAST	basic local alignment search tool
B. juncae	*Brassica juncea*
B. napus	*Brassica napus*
bp	base pair
BnDIR1	*Brassica napus DIR 1*

B. nigra	*Brassica nigra*
BnNPR1	*Brassica napus NPR 1*
BnPR1	*Brassica napus Pathogenesis Related 1*
BnPR2	*Brassica napus Pathogenesis Related 2*
B. oleracea	*Brassica oleracea*
B. rapa	*Brassica rapa*
BSA	bovine serum albumin
BTB/POZ	broad-complex, tramtrack, and bric-a-brac/pox virus and zinc finger
BTH	benzo (1,2,3) thiadiazole-7-cabothionic acid S-methyl ester
bZIP	basic leucine zipper
CAMBIA	Center for the Application of Molecular Biology to International Agriculture, CAMBIA, Inc., Canberra, ACT, Australia
CaMV	Cauliflower Mosaic Virus
CaMV35S	Cauliflower Mosaic Virus 35S promoter
cDNA	complementary DNA
cfu	colony forming units
ChIP	chromatin immunoprecipitation
CMV	Cucumber Mosaic Virus
Col-0	*A. thaliana* ecotype Columbia
dATP	deoxy adenosine triphosphate
DB	DNA-binding domain
DHAP	dihydroxyacetone phosphate
DIR1	*DEFECTIVE IN INDUCED RESISTANCE 1*
DNA	deoxyribonucleic acid
E	Einstein units
E. cichoracearum	*Erysiphe cichoracearum*
eds	*enhanced disease susceptibility*
EDTA	ethylenediamine-tetraacetic acid
EST	expressed sequence tag
ET	ethylene

EtBr	ethidium bromide
ETR1	*ETHYLENE RECEPTOR 1*
FAO	Food and Agriculture Organization of the United Nations
F. oxysporum	*Fusarium oxysporum*
g	gram(s)
GC/MS	gas chromatography/mass spectrometry
GFP	green fluorescent protein
GST-6	*GLUTATHIONE S-TRANSFERASE 6*
GUS	β-glucuronidase
HPT	hygromycin phosphotransferase
H. parasitica	*Hyaloperonospora parasitica*
HR	hypersensitive response
h	hour(s)
ICS	*isochorismate synthase 1*
IFR	*isoflavone reductase*
INA	2,6-dichloroisonicotinic acid
ISR	induced systemic resistance
JA	jasmonic acid
JAR1	*JASMONIC ACID RESPONSIVE 1*
l	litre(s)
LacZ	β-galactosidase gene
ld	leaf disc
L. esculentum	*Lycopersicum esculentum*
L. maculans	*Leptosphaeria maculans*
LPS	lipopolysaccharides
LRR	leucine rich repeats
LS	linker scan
LTP	lipid transfer protein
K	kilo-
m	milli-, meter(s)
M	molar

MeJA	methyl jasmonate
MeSA	methyl salicylate
$MgCl_2$	magnesium chloride
min	minute(s)
ml	milliliter
mm	millimeter
mRNA	messenger RNA
MS	Murashige and Skoog
MSB	menadione sodium bisulphate
mol	moles
MUG	methyl-umbelliferyl-β-D-glucuronide
m/z	mass per unit charge
NahG	*salicylate hydroxylase*
NBS	nucleotide binding site
NDR1	*NON-RACE SPECIFIC DISEASE RESISTANCE 1*
NH1	*NPR1* homolog 1
NIM1	*NON-INDUCIBLE IMMUNITY 1*
NIMIN	*NIM1 INTERACTING PROTEIN 1*
NLS	nuclear localizing signal
nos	*nopaline synthase*
N:P:K	nitrogen:phosphorus:potassium
NPR1	*NON-EXPRESSER OF PATHOGENESIS-RELATED GENES 1*
nptII	*neomycin phosphotransferase II*
N. tabacum	*Nicotiana tabacum*
ocs	*octopine synthase*
O^{18}	oxygen isotope
OD_x	absorbance at wavelength (x)
O. sativa	*Oryza sativa*
PAD4	*PHYTOALEXIN DEFICIENT 4*
PAL	*PHENYL AMMONIA LYASE*
PAMPs	pathogen-associated molecular patterns

PCA	protein fragment complementation assay
PCD	programmed cell death
PCR	polymerase chain reaction
P. infestans	*Phytophthora infestans*
PR	pathogenesis-related
PROSITE	database of protein families and domains
Psm	*Pseudomonas syringae* pv. *maculicola*
Pst	*Pseudomonas syringae* pv. *tomato* DC3000
pv	pathovar
RACE	random amplification of cDNA ends
R-gene	*Resistance-gene*
RNA	ribonucleic acid
RNAi	RNA interference
R. solanacearum	*Ralstonia solanacearum*
R. solani	*Rhizoctonia solani*
SA	salicylic acid
SABP2	salicylic acid-binding protein 2
SAG	SA 2-O-β-D-glucoside
SAG101	*SENESCENCE-ASSOCIATED GENE 101*
SAI1	*SALICYLIC ACID INSENSITIVE 1*
SAR	systemic acquired resistance
SDS	sodium dodecyl sulphate
sec	second(s)
SFD1	*SUPPRESSOR OF FATTY ACID DESATURASE DEFICIENCY 1*
SID2	*SALICYLIC ACID DEFICIENT 2*
S. sclerotiorum	*Sclerotinia sclerotiorum*
S. solani	*Stemphylium solani*
TAE	Tris, sodium acetate, and EDTA
tCUP	tobacco constitutive promoter
TCV	Turnip Crinkle Virus
T-DNA	*Agrobacterim tumefaciens* transfer-DNA

TE	Tris buffer and EDTA
TILLING	Targeting Induced Local Lesions In Genomes
TMV	Tobacco Mosaic Virus
TNV	Tobacco Necrosis Virus
Tween 20	polyoxyethylene sorbitan monolaurate
TYLCV	Tomato Yellow Leaf Curl Virus
uidA	β-glucuronidase gene (GUS)
VIGS	virus-induced gene silencing
v/v	volume/volume
Ws	*Brassica napus* cultivar Westar
Ws	*Arabidopsis thaliana* ecotype Wassilewskija
wt	wild-type
X. campestris	*Xanthomonas campestris*
X-Gluc	5-bromo-4-chloro-3-indolyl-ß-D-glucuronic acid cyclohexyl ammonium salt
X. oryzae	*Xanthomonas oryzae* pv. *oryzae*
X-GAL	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
2YT	growth medium containing yeast extract and bacto- tryptone

CHAPTER 1. Literature Review

1.1. General introduction

Many plants are the longest living higher organisms on the planet. They have evolved various sophisticated and effective mechanisms to recognize and combat pathogenic microorganisms when attacked, and have thrived over millions of years in spite of their immobility. Disease resistance is broadly defined as the plant's ability to suppress the damaging effect of a pathogen. Plants contain genetic information required to resist infection from a plethora of pathogenic organisms including viruses, bacteria, oomycetes and fungi. They exhibit both short- and long-term defense responses when attacked by pathogens and deploy various defense mechanisms including tissue reinforcement at the site of infection, production of various anti-microbial compounds and induction of defense-associated genes and proteins (Agrios, 1997).

Plant-microbial interactions are very complex and involve a two-way communication at the molecular level. The success of the plant however depends on the intensity and expeditious perception of signals from the pathogen and their transmission between and within plant cells to produce an effective response against the pathogen. Knowledge of the underlying mechanisms involved in such defense responses helps the basic understanding of plant-pathogen interactions and can be exploited to produce improved disease resistance in crops.

This study investigated a type of broad range, inducible defense response known as systemic acquired resistance (SAR; reviewed in section 1.6.1) in the crop plant *Brassica napus* (canola), with particular focus on the molecular biology of SAR regulation and the consequences of overexpressing known or putative regulatory genes on resistance to disease.

1.2. Disease resistance in plants

Plants are constantly exposed to pathogenic microorganisms and the diseases they cause lead to billions of dollars in agricultural losses per annum world-wide (FAO, 2004;

Strange and Scott, 2005). To combat pathogens, plants have developed an array of passive and active defense mechanisms (Hammerschmidt, 1999). These are either constitutive or inducible, physical or chemical (Lamb et al., 1989; Lamb, 1994). Some plants produce substances such as waxes, cutin, suberin, lignin, calcium and silicon that can present structural physical barriers to penetration by the invading pathogen. The presence of preformed antimicrobial compounds, including secondary metabolites such as tannins and saponins (Osbourn, 2003) and cationic peptides (Broekaert et al., 1995) serve as constitutive chemical defenses.

Pre-existing mechanisms do not always sufficiently protect plants against pathogen attack. Therefore plants have developed active defense mechanisms. Some of the inducible defenses include cross-linking of cell wall components to form polyphenolics and the deposition of the β-1,3 glucan callose, rendering the plants resistant to hydrolytic enzymes that are released by infecting pathogens (Heath, 2000; Richter and Ronald, 2000). Secondary metabolites (phytoalexins) and pathogenesis-related (PR) proteins having antimicrobial properties are also induced following pathogen recognition (Sticher et al., 1997).

The activation of inducible plant defenses is mediated by plant growth regulators, including salicylic acid (SA), jasmonic acid (JA) and ethylene (ET; Ton et al., 2002; Beckers and Spoel, 2006). SA-dependent signal transduction pathways are required for resistance to certain pathogens that derive energy from living host cells (i.e. biotrophs; Glazebrook, 2005). JA and ET signaling are generally required for resistance to necrotrophic pathogens (i.e. derive energy from killed cells). The SA- and JA/ET-dependent signaling pathways appear to interact in a complex fashion, with the primary mode of interaction being mutual antagonism (Feys and Parker, 2000). Inter-pathway communication has been speculated to help plants fine-tune and prioritize defense responses upon encountering multiple signals (Beckers and Spoel, 2006).

1.3. Non-host resistance

The most effective type of disease resistance is non-host resistance that is manifested when a pathogen comes in contact with a plant on which it cannot cause disease and therefore the plant is completely protected from infection (Agrios, 1997; Heath 2000). An important criterion in defining non-host resistance is that all individuals

in a plant species must be resistant to all isolates of a pathogen species. Non-host inducible defense responses are triggered by the recognition of common molecular features that are thought to be absolutely required for pathogenicity, referred to as pathogen-associated molecular patterns (PAMPs; Zipfel et al., 2004). Examples of PAMPs include flagellin, the main protein component of bacterial flagella, peptidoglycan of Gram positive bacteria, lipopolysaccharide of Gram-negative bacteria, double stranded RNA of some viruses, and unmethylated DNA. In cases where individuals within a plant species are resistant to some, but not all, isolates of a pathogen, the resistance is said to be host-specific.

1.4. Race-specific resistance

Race-specific resistance is a well-studied example of host-resistance and has been observed between plants and a diverse array of pathogens, including viruses, bacteria, fungi, nematodes, and insects (Crute, 1986). The genetic interrelationship between plant and microbe during this type of interaction is described by the gene-for-gene hypothesis, first proposed by Flor in 1942. Disease resistance is determined by the presence of dominant *Resistance* (*R*) genes in the plant and corresponding dominant *Avirulence* (*Avr*) genes in the pathogen (Staskawicz et al., 1995; Van Der Biezen and Jones, 1998). Such interactions are said to be incompatible and are characterized by rapid calcium and other ion fluxes, an extra cellular oxidative burst, transcriptional reprogramming within and around the infection sites and, in most cases, a localized programmed cell death (PCD), known as the hypersensitive response (HR) (reviewed in Bonas and Lahaye, 2002). A combination of all these responses is thought to contribute to stopping the growth of the pathogen.

In the absence of corresponding *R*-gene – Avr factor combinations (i.e. if the plants lack *R* genes corresponding to Avr factors produced by the pathogen) defense responses are not effectively deployed and disease ensues (Staskawicz et al., 1995). The plant is said to be susceptible, the pathogen is virulent and the interaction is compatible. Several *R* genes have now been isolated and can be broadly classified into three classes based on the proteins they encode (Schornack et al., 2006). These are detoxifying enzymes, intracellular protein kinases and proteins containing leucine rich repeats (LRR), with the majority of R proteins characterized to date falling into the last class. LRR

domains are found in proteins with diverse functions and have been implicated in interactions between proteins, carbohydrates and other ligands (Jones and Jones, 1996; Kobe and Kajava, 2001). These can act as specificity determinants for pathogen recognition (Jones and Jones, 1996) and may be involved in signaling (Banerjee et al., 2001). Cytoplasmic LRR-containing R proteins also contain a conserved nucleotide binding site (NBS) that probably binds ATP or dATP (Tameling et al., 2002). This subclass of R proteins may be further divided depending upon the presence of either an N-terminal coiled-coil domain (CC-NBS-LRR) or Toll-interleukin 1 homology domain (TIR-NBS-LRR). It is predicted that the *Arabidopsis thaliana* (L.) Heynh (hereafter referred to as *Arabidopsis*) genome may contain ~150 NBS-LRR proteins that could confer resistance to pathogens and pests as diverse as viruses, bacteria, fungi, nematodes, and aphids (Eckardt and Innes, 2003; Meyers et al. 2003).

The *R* genes were initially thought to encode extra cellular receptor-like proteins (Martin, 1999; Schornack et al., 2006). This is true for some *R* genes, but many others encode intracellular proteins. The discovery of the bacterial type III secretion system (TTSS), provided evidence that pathogen recognition could occur within the cell (Alfano and Collmer, 1997). Many phytopathogenic bacteria inject effector proteins directly into plant cells via a Hrp (HR and pathogenicity) TTSS. This system helps the pathogens to suppress plant defenses, grow in plants and produce disease lesions in hosts (Alfano and Collmer, 2004). The TTSS is known to involve approximately 20-25 different proteins and requires ATPase as well as transmembrane ionic potential (Ghosh, 2004). *Pseudomonas syringae* pathotype (a classification of pathogens that distinguish them from other members of the same species by their pathogenicity on a specific host or hosts; pv.) *tomato* (*Pst*) DC3000 is a widely studied plant pathogen that causes disease on several plant species. This pathogen also uses TTSS to directly deliver effector proteins into the host cell (Galan and Collmer, 1999). Loss-of-function mutations in the TTSS abolish disease formation, indicating that effectors are essential agents of *Pst* pathogenesis (Collmer et al., 2000).

Although it was initially predicted that R proteins would act as receptors of Avr factors, there has been limited evidence to support direct interactions between R proteins (in particular those containing LRR) and corresponding Avr factors (Bonas and Lahaye,

2002; Dangl and Jones, 2001; Martin et al., 2003). In the absence of such evidence, it has been proposed that R proteins recognize the consequences of Avr factor action, rather than the factors themselves (Dangl and Jones, 2001). According to the guard hypothesis, Avr factors modify targets within plant cells: it is these changes in the plant targets that R proteins recognize, leading to the initiation of signaling events required to establish resistance to disease. Conceptually, R proteins can be thought to "guard" these plant targets. The guard hypothesis has yet to be conclusively proven, but provides a reasoning as to why R proteins are not the direct targets of Avr proteins.

The consequence of gene-for-gene interaction often leads to the establishment of broad range resistance to otherwise virulent pathogens. This special type of resistance is called systemic acquired resistance (SAR; Ryals et al., 1996) and is reviewed in a separate section below (section 1.6.1.).

1.5. Basal defense

As stated above, if the pathogen does not contain an *Avr* gene that can be recognized by the host (compatible interaction), the plant reacts either too late or inefficiently to stop pathogen growth and disease ensues. Although resistance against these virulent pathogens is considerably less when compared to avirulent pathogens, the plant still executes strategies to limit colonization by the virulent pathogen. This resistance has been referred to as basal resistance and has been demonstrated by the isolation of mutants that are hypersusceptible to otherwise virulent pathogens (Glazebrook, 2001; Durrant and Dong, 2004). Basal defenses have been shown to overlap with *R*-gene mediated resistance but are temporally slower and of lower amplitude (Dangl and Jones, 2001; Ausubel, 2005).

1.6. Induced disease resistance

Induced disease resistance is a phenomenon that is activated after appropriate stimulation of the plants either with a biological agent, such as a microbe, or a chemical agent resulting in predisposing the plant to resist further pathogen attack (Hammerschmidt, 1999). Induced disease resistance mechanisms are active, energy-requiring systems typified by specific recognition of a biological agent that leads to a series of physiological changes providing both local and systemic resistance to the

pathogen. It can include the development of an HR, production of PR proteins, synthesis of antimicrobial phytoalexins and reinforcement (lignification) of the plant cell wall.

Induced resistance triggered by biological agents can be broadly divided into two categories, namely SAR and induced systemic resistance (ISR, see section 1.6.2). A variety of chemicals such as SA, and the SA analogs benzo (1,2,3) thiadiazole-7-cabothionic acid S-methyl ester (BTH) and 2,6-dichloroisonicotinic acid (INA) have been shown to trigger induced resistance, most of them through the SAR response (Ryals et al., 1996). The non-protein amino acid BABA (β-amino butyric acid) has been shown to induce disease resistance through a pathway that is distinct from SAR and ISR (Cohen, 2002; Jakab et al., 2001).

There is evidence that simultaneous activation of multiple induced resistance pathways (e.g. SAR and ISR) results in enhanced levels of protection against pathogens, suggesting that defense responses activated through different pathways are additive (Van Wees et al., 2000).

1.6.1. Systemic acquired resistance

Frank Ross (1961, Cornell University) was the first to provided a detailed description of the SAR phenomenon. He demonstrated that tobacco plants showing HR were able to develop enhanced disease resistance in non-inoculated leaves against subsequent infection by Tobacco Mosaic Virus (TMV). Local pre-inoculation with avirulent strains of *Pseudomonas syringae* has also been shown to induce SAR to several virulent pathotypes of this bacterial pathogen in *Arabidopsis* (Alvarez et al., 1998; Cameron et al., 1994), while pre-inoculation with TMV or oomycete *Peronospora tabacina* pv. *tabacina* induced resistance against TMV and *P. tabacina* fungi and viruses in tobacco (Ozeretskovskaya, 1995) and pre-inoculation with the fungus *Colletotrichum lindemuthianum* in *Phaseolus vulgaris* induced resistance against *C. lindemuthianum* and *Uromyces appendiculatus* (Dann and Deverall, 1995). This suggests that SAR can be induced by a broad range of biological pathogens. SAR has now been documented as an effective defense response in a variety of plant species including monocots and dicots, against a broad range of pathogens including viruses, bacteria, oomycetes and fungi (Durrant and Dong, 2004; Kuć, 1982; Ryals et al., 1996; Sticher et al., 1997).

Many SAR studies have been performed with three model systems. Earlier studies focused on interactions between tobacco and TMV (Ross, 1961) and *P. tabacina* (Ozeretskovskaya, 1995) as well as cucumber infected by *Colletotrichum* spp., Tobacco Necrosis Virus (TNV) and *Pseudomonas* spp. (Hammershmidt and Yang-Cashman, 1995). In the last fifteen years, *Arabidopsis* has emerged as the model plant to study the molecular biology of SAR (Ryals et al., 1997). In this species, SAR has been shown to be inducible by avirulent strains of *P. syringae*, *Fusarium oxysporum*, and Turnip Crinkle Virus (TCV), and effective against *Pst* DC3000, *P. syringae* pv. *maculicola* (*Psm*) the oomycete *Hyaloperonospora parasitica* (formerly known as *Peronospora parasitica*; Constantinescu and Fatehi, 2002) and TCV (Sticher et al., 1997). SAR is not effective against all pathogens; notable exceptions include the lack of protection of tobacco against two of nine pathogens tested, *Botrytis cinerea* and *Pst* DC 3000 (Friedrich et al., 1996), and the inability to immunize cucurbits against *Uncinula necator* which causes powdery mildew (Kuć, 1982).

Studies in cucumber and tobacco (Kuć, 1982) and later in *Arabidopsis* suggest that SAR consists of three main stages (Cameron et al., 1994; Wolfe et al., 2000). The first stage, initiation or immunization, is triggered by localized exposure to microbes that cause plant cell death, either a HR as part of an incompatible reaction (see section 1.4) or disease-associated necrosis (Ryals et al., 1996). This leads to the accumulation of SA (10-15 fold increase over background levels; Yalpani et al., 1991) and expression of *PR* genes (Uknes et al., 1993). The initiation stage is also accompanied by the production of a mobile signal that is thought to move via the phloem from the induced leaf to the rest of the plant to establish SAR. The requirements for SA, association with *PR* genes and nature of the mobile signal are all discussed in more detail in the following sections. It is noteworthy that there are some examples in which SAR occurs without visible necrosis (Cameron et al., 1994; Keller et al., 1996).

The second stage of SAR is the establishment stage which involves the perception of the mobile signal in systemic, non-infected leaves resulting in the occurrence of low frequency microscopic HRs (Alvarez et al., 1998). It is characterized by the expression of the same set of *PR* genes induced around the primary necrotic lesion, as well as the accumulation of SA, although to much lower levels than are induced in the primary leaf

during the induction stage (1-2 fold in *Arabidopsis*, Cameron et al., 1999; 10 fold in tobacco, Yalpani et al., 1991).

The final stage in the SAR pathway is the expression or manifestation stage that occurs when the plant is challenged with a second, normally virulent pathogen and responds to that pathogen by displaying resistance. This response involves the rapid production of PR proteins, accumulation of SA, increased lignification and even suppression of pathogen virulence factor production (Cameron et al., 1999; Kuć, 1982; Siegrist et al., 1994; Ye et al., 1989; Palva et al., 1994).

The time needed for the establishment of SAR depends on both the plant and the inducing organisms. A very rapid induction was reported for cucumber, where SAR sets in as early as 7 h after primary inoculation with *P. syringae* (Smith et al., 1991). Injection of spores of the blue mold pathogen, *P. tabacina* under the epidermis of the stem of tobacco plants leads to the expression of SAR in the leaves against the same fungus 2-3 weeks after the primary inoculation (Cohen et al., 1987). The level of protection may vary depending on the organism used for the primary inoculation and particularly on the extent of necrosis (Madamanchi and Kuć, 1991). The type of necrosis and its time of development are critical. For example, wounds inflicted after contact with hot or very cold objects appear within hours but generally do not induce SAR (Madamanchi and Kuć, 1991). Similarly, wounding from syringe inoculation of $MgCl_2$ in *Arabidopsis* does not induce SAR (Cameron et al., 1999).

Once established, SAR can last up to several weeks (Hammerschmidt and Kuć, 1995). In cucumber, inoculation of the first leaf, followed 2-3 weeks later by a second booster inoculation, protects plants up to flowering (Madamanchi and Kuć, 1991). SAR is also effective under field conditions as demonstrated in trials with bean, tobacco, and cucumber (Sutton, 1982).

1.6.1.1. Role of SA

Plants, unlike animals, can synthesize SA and activate SA-dependent physiological programs (Klessig and Malamy, 1994). Two different SA biosynthetic pathways have been described so far. The first involves synthesis of SA from chorismate using isochorismate synthase 1, which is encoded by the pathogen-inducible gene *SALICYLIC ACID DEFICIENT2* (*SID2*; Wildermuth et al., 2001). *Arabidopsis* mutants at

the *sid2* locus accumulate only 5-10% of wild-type SA levels following pathogen infection and are compromised in basal resistance and SAR (Wildermuth et al., 2001). *sid2* is allelic to *enhanced disease susceptibility16* (*eds16*) that was isolated in a screen for mutants hypersusceptible to the fungal pathogen *Erysiphe orontii* and contained reduced levels of SA after infection (Dewdney et al., 2000). These results suggest that the isochorismate pathway might be the major route to defense-associated SA production in *Arabidopsis* (Shah, 2003). SA synthesis may also be catalyzed by phenylalanine ammonia-lyase (PAL) from cinnamic acid as a substrate (Ribnicky et al., 1998; Yalpani et al., 1993). There is evidence that PAL is required for SAR in tobacco (Pallas et al., 1996).

A large fraction of the SA pool is present in the form of its conjugates, derived from glucosylation or esterification of the unique hydroxyl or carboxyl groups, or from modifications in other positions of the aromatic ring (Enyedi et al., 1992; Klessig and Malamy, 1994). The main conjugate in tobacco is SA 2-O-β-D-glucoside (SAG), which accumulates in the vicinity of HR lesions upon the activation of SA glucosyltransferase. This enzyme is inducible when high concentrations of SA are reached in infected tissues (Enyedi et al., 1992).

Two different groups, Malamy et al. (1990) and Métraux et al. (1990), discovered that SA levels increased in plants undergoing SAR. The spatiotemporal pattern of SA accumulation during the HR suggests that SA is implicated in controlling the timing and extent of cell death and subsequent *PR* gene expression (Dangl et al., 1996; Greenberg, 1997). These observations led to the hypothesis that SA acts as a signal that triggers SAR. This model was supported by experiments using transgenic tobacco plants expressing the *Pseudomonas putida NahG* gene encoding a salicylate hydroxylase that converts SA to catechol. Plants expressing this gene were unable to accumulate SA, and importantly were unable to mount a SAR response (Gaffney et al., 1993). *NahG* transgenics have subsequently been generated and tested in a number of plant species. Similar to tobacco plants described above, *Arabidopsis NahG* plants were compromised in the induction of *PR* genes and were more susceptible to a broad range of pathogens including viruses, bacteria, fungi and oomycetes (Delaney et al., 1994; Kachroo et al., 2000). These plants also permitted growth of normally incompatible races of both fungal

and bacterial pathogens, indicating that SA plays a role in mediating basal and race-specific resistance. It is noteworthy that some of the effects observed in *NahG* plants were recently shown to be non-specific metabolic effects of the transgene (Heck et al., 2003; Van Wees and Glazebrook, 2003). For example, it was shown that catechol (degradation product of SA) rather than low amount of SA was responsible for the loss in non-host resistance of *NahG* plants (Van Wees and Glazebrook, 2003).

Further evidence for the role of SA in mediating plant defense responses comes from the analysis of the *eds5* mutant, also known as *sid1*. *sid1* was identified in a screen based on impaired accumulation of SA after pathogen infection and found to display enhanced susceptibility to the virulent pathogens *Pst* and *H. parasitica* (Nawrath and Métraux, 1999). *eds5* was identified as a mutant hypersusceptible to a virulent strain of the bacterial pathogen *Xanthomonas campestris* pv. *raphani* (Rogers and Ausubel, 1997). The predicted product of *EDS5* is homologous with members of the MATE (multidrug and toxin extrusion) transporter family and contains a series of nine to 11 membrane-spanning domains and a coil domain at the N terminus.

It is noteworthy that *eds5/sid1* and *sid2/eds16* mutants are not as susceptible to pathogens as are transgenic plants expressing *NahG*, and that only the expression of *PR-1* is strongly reduced in these mutants after pathogen attack (Nawrath and Métraux, 1999). In contrast *PR-2* and *PR-5* are also reduced in *NahG* plants. Furthermore, accumulation of the phytoalexin camalexin was only affected in *NahG* plants (Nawrath and Métraux, 1999), and patterns of JA and ET following pathogen challenge differ between *NahG* plants and *eds5/sid1* and *sid2/eds16* mutants (Heck et al., 2003). Since SA levels following pathogen challenge in *eds5/sid1* and *sid2/eds16* mutants are similar to those in *NahG* transgenics (Nawrath and Métraux, 1999), it has been speculated that some of the effects observed in *NahG* plants may not be directly attributed to reduced levels of SA (Heck et al., 2003; van Wees and Glazebrook, 2003). Indeed, van Wees and Glazebrook (2003) observed that only *NahG Arabidopsis*, and not *eds5/sid1* or *sid2/eds16* mutants were compromised in non-host resistance to *P. syringae* pv. *phaseolicola*. Interestingly, resistance was not compromised in *sid2 NahG* double mutants. Since these double mutants do not accumulate SA, they do not produce substrate for salicylate hydroxylase, suggesting that the phenotype observed in the *NahG* transgenics may be attributed to one

or more products of SA degradation by this enzyme (van Wees and Glazebrook, 2003). This notion was substantiated by the observation that exogenous application of catechol, the immediate degradation product of SA by salicylate hydroxylase, compromised resistance of wild-type plants against *P. syringae* pv. *phaseolicola* (van Wees and Glazebrook, 2003). In contrast, the *sid2 NahG* double mutant behaves as the *NahG* (*SID2*+) transgenic with respect to JA and ET accumulation (Heck et al., 20003), suggesting that these *NahG* phenotypes may not be attributed to the production of catechol (Heck et al., 2003). Instead, these authors speculated that the *P. putida* salicylate hydroxylase may act on substrates other than SA. Finally, Cameron (2000) raised the possibility that *NahG* plants may alter the flux through the phenylpropanoid pathway in an attempt to compensate for their inability to accumulate SA, with the predicted consequence being reduced levels of anti-microbial phenolics and lignin. The above scenario remains to be experimentally confirmed.

A third line of evidence implicating SA in the control of SAR is that the exogenous application of this metabolite, or its functional analogs INA, BTH and others, are potent inducers of disease resistance (Ryals et al., 1996). To date, SA is the only plant-derived substance that has been demonstrated to be an inducer of SAR (White, 1979; Antoniw and White, 1980; Ward et al., 1991). The chemical INA was the first synthetic compound shown to activate SAR (Vernooij et al., 1995). But, SA and INA were not tolerated by crop plants due to their toxic effects and therefore could not be used in practical applications in the fields. In contrast, BTH was shown to be a potent activator of SAR that provides resistance against a broad range of plant pathogens (Friedrich et al., 1996; Görlach et al., 1996; Lawton et al., 1996). Therefore BTH is an attractive compound for practical uses in agriculture, and BTH formulations are sold as BION® or Actiguard (Syngenta, Basel, Switzerland).

Neither SA, INA or BTH exhibit direct antimicrobial activity. However, they trigger the same spectrum of differential gene expression observed following biological SAR induction (Maleck et al., 2000) suggesting that their mode of action is by activating this plant defense response. Furthermore, these chemicals induce resistance against the same spectrum of pathogens as does biologically-activated SAR (Friedrich et al., 1996). The exact function of SA is still unknown (Cameron, 2000). Information regarding the

cellular and sub-cellular localization of SA during different stages of SAR might help further elucidate its role during SAR. Recent studies have shown that SA regulates the activity of proteins implicated in the regulation of SAR, namely TGA factors and NPR1 (see section 1.7.1).

1.6.1.2. Nature of the systemic signal

Based on girdling experiments (Gianinazzi and Ahl, 1983; Guedes et al., 1980), it was proposed that the generated systemic signal for SAR was phloem-mobile. SA was initially proposed as a candidate for the signal, since it accumulates in the phloem (Malamy et al., 1990. Métraux et al., 1990, Yalpani et al., 1991) and O^{18} labeling experiments support SA synthesis and movement following infection (Shulaev et al., 1995). However, leaf detachment experiments in cucumber (Rassmussen et al., 1991) and grafting experiments with transgenic *NahG* (Vernooij et al., 1994) and *PAL* sense suppressed (Pallas et al., 1996) tobacco suggested that SA is not the phloem-mobile signal (Gaffney et al., 1993; Pallas et al., 1996). Of note, the grafting experiments demonstrated that even if leaves on one side of the graft were unable to produce SA, they were still capable of inducing SAR on the other side of the graft, strongly arguing for the presence of a signal other than SA to induce SAR. A number of studies suggest that a non-SA and/or phloem mobile signal may move from the necrotic leaf to the rest of the plant to establish SAR (Guedes et al., 1980; Kiefer and Slusarenko, 2003; Maldonado et al., 2002, Rasmussen et al., 1991; Tuzan and Kuć, 1985). The analysis of the *Arabidopsis defective in induced resistance 1-1* (*dir1-1*) mutant has led to the hypothesis that a lipid may be a component of the long distance SAR signal (Maldonado et al., 2002; reviewed in section 1.7.2).

1.6.1.3. Pathogenesis-related genes

SAR is also associated with the accumulation of gene transcripts and proteins, sometimes referred to as SAR genes and proteins. This may be observed locally at the site of infection or at the non-infected sites of the plant (Uknes et al., 1992; Ward et al., 1991). Analysis of *SAR* genes showed that many of them encode previously known PR proteins which have a wide range of structures and functions. At least some have been shown to possess antimicrobial properties, for example, chitinases (e.g. *PR-3*), glucanases

(e.g. *PR-2*) and cationic peptides (Van Loon and Van Strien, 1999). The function of many PR proteins is still unknown.

Nine different families of mRNA encoding SAR proteins were shown to be correlated with the onset of SAR (Ward et al., 1991). These gene families include PR proteins PR-1 (PR-1a, PR1-b and PR1-c), PR-2 (β-1,3-glucanse; PR-2a, PR2-b and PR-2c), PR-3 (class II chitinase; PR-3a and PR-3b), PR-4 (hevein-like protein; PR-4a and PR-4b), PR-5 (thaumatin-like protein; PR-5a and PR-5b), acidic and basic isoforms of class III chitinase, an extracellular β-1,3-glucanase (PR-Q'), and the basic isoform of PR-1. In *Arabidopsis*, there are three well recognized SAR genes namely, *PR-1*, *PR-2* and *PR-5* (Uknes et al., 1992). The repertoire of SAR genes varies in different plant species, perhaps due to different pathogen landscapes during the evolution of the plant species. Also, the relative expression levels of *PR* genes vary between different plant species. For example, in *Arabidopsis* and tobacco, acidic *PR-1* gene is expressed most predominantly, whereas in cucumber it is expressed to very low levels (Ryals et al., 1992). Due to the characteristic expression of *PR* genes during SAR, they have been used as molecular markers. For example in *Arabidopsis*, *PR-1* has been widely used as a marker gene for SAR.

In addition to known *PR* genes there are several other genes that are up- or down-regulated during SAR or in response to SA (Maleck et al., 2000; Pan et al., 2004; Pylatuik and Fobert, 2005). However, the function of most of these genes is still unknown (Eulgem, 2005).

1.6.1.4. Manifestation of SAR

In addition to the protective effects of PR proteins, SAR has also been shown to be associated with cross-linking of cell wall proteins and the deposition of lignin and callose at the site of secondary pathogen attack (Richter and Ronald, 2000). Callose deposition contributes to disease resistance by reinforcing the plant cell wall beneath fungal penetration sites (Kauss, 1992). Furthermore, BTH-pretreated *Arabidopsis* plants show stronger accumulation of *PAL* and/or enhanced callose deposition upon *Pst* DC3000 infection. PAL is a key enzyme in the phenylpropanoid pathway that leads to a variety of defense-related plant secondary metabolites such as phytoalexins and lignin-like polymers (Hahlbrock and Scheel, 1989). Although SA can be produced by PAL as

described earlier (section 1.6.1.1), SA required for SAR in *Arabidopsis* is majorly produced by isochorismate synthase (ICS) pathway and not the PAL pathway (Wildermuth et al., 2001).

1.6.2. Induced systemic resistance

Induced systemic resistance (ISR) is a biologically-induced resistance that is developed in response to colonization of roots by non-pathogenic rhizobacteria (Van Peer et al., 1991; Wei et al., 1991). ISR has been demonstrated in different plant species and offers resistance against a wide range of pathogens (Van Loon et al., 1998; Ton et al., 2002). It is activated by LPS and flagella of some non-pathogenic *Pseudomonas* strains involving an SA-independent signaling pathway (Felix et al., 1999; Van Loon et al., 1998; Van Wees et al., 1997). Although the exogenous application of flagella or purified LPS can induce ISR, bacterial mutants lacking flagella or the O-antigenic side chain of the LPS were still able to elicit ISR in *Arabidopsis* indicating that more determinants may be involved in the elicitation of rhizobacteria-mediated ISR (Van Wees et al., 1997).

The existence of an SA-independent ISR pathway was first demonstrated in *Arabidopsis* using *P. fluorescens* strain WCS_{417r} as the ISR-inducing agent and *Pst* DC3000 as the secondary challenging pathogen (Pieterse et al., 1996). It was shown that ISR was functional in the SA deficient *NahG* plants and is not associated with *PR* genes that are activated after SA treatment. The JA-insensitive *jar1* (*jasmonic acid responsive 1*) or the ET-insensitive *etr1* (*ethylene receptor 1*) mutant plants fail to trigger ISR indicating that both JA and ET-response pathways are required for the establishment of ISR (Pieterse et al., 1998). Furthermore, using methyl jasmonate (MeJA) and 1-aminocyclopropane-1-carboxylate (ACC) as ISR activators, it was shown that JA signaling functions upstream of ET in the signaling pathway.

1.6.3. BABA induced resistance

A third type of induced resistance is activated upon treatment with a non-protein amino acid, β-aminobutyric acid (BABA; Cohen and Gisi, 1994; Jakab et al., 2001; Zimmerli et al., 2000). It was demonstrated that BABA-induced resistance (BABA-IR) protected *Arabidopsis* against *H. parasitica* by activating the plant's natural defense mechanisms such as callose deposition and HR (Zimmerli et al., 2000). They also showed that this induced defense was not inhibited by mutants impairing the SA-, JA-, or ET-

response pathways, suggesting that BABA resistance functions independently from ISR and SAR. BABA-IR is effective against both biotrophic and necrotrophic pathogens (organisms that derive energy from living and dead cells, respectively) (Ton and Mauch-Mani, 2004), as well as certain types of abiotic stress (Cohen, 2002; Jakab et al., 2005; Zimmerli et al., 2001). BABA-IR is not associated with direct activation of defense-related genes (Jakab et al., 2001; Van Loon et al., 1998).

1.6.4. Priming during induced resistance responses

Although SAR, ISR and BABA-IR are induced by different agents, involve different signaling molecules and are associated with various kinds of defense proteins, a common feature that ties them together is the capacity for priming (Conrath et al., 2001). Priming was first discovered in plant suspension cultures by Kauss et al. in 1992 and is often associated with an enhanced capacity to mobilize infection-induced, cellular defense responses. Although the phenomenon has been known for years, major progress in the understanding of priming was made only recently.

In *Arabidopsis*, it was shown that after infection with *P. syringae*, the plants expressed increased levels of defense related genes such as *PAL* (Kohler et al., 2002). Inoculation of *Arabidopsis* with ISR-inducing agent *P. fluorescens* WCS_{417r} did not directly activate defense-related genes. However, *Arabidopsis* plants were primed for enhanced expression of JA- and ET-inducible genes upon infection by *P. syringae* (Van Wees et al., 1999; Verhagen et al., 2004). Treatment of *Arabidopsis* with BABA leads to priming of SA-dependent defences (Zimmerli et al., 2001) including enhanced formation of callose-rich papillae that functions independently from SA (Ton and Mauch-Mani, 2004). Recently, Ton et al. (2005) showed that these forms of priming require a different set of signaling components involving priming alone.

1.7. Genetic regulators of SAR

During the last few years, *Arabidopsis* has become a very important model plant to unravel how defense systems can control pathogen attack. This is largely due to the availability of many mutants in different defense response pathways. Studies using these mutants have led to a reasonable insight as to how different defense pathways interact to control various pathogens (Figure 1.1; Glazebrook, 2005).

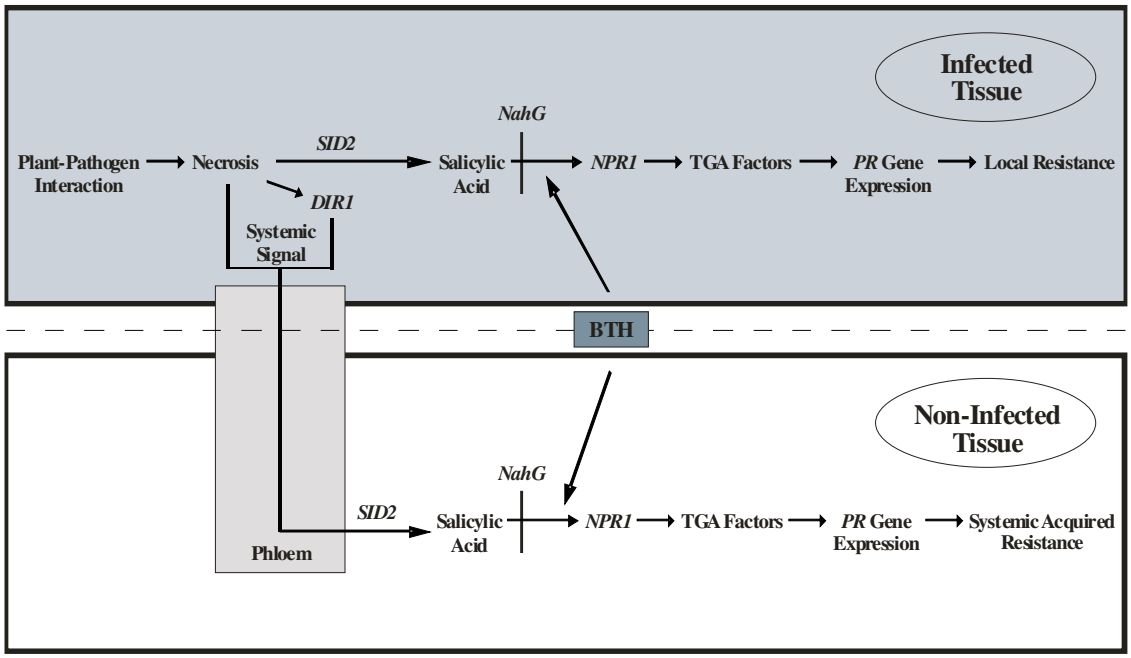


Figure 1.1. General scheme of signal transduction pathways involved in systemic acquired resistance (modified from Ryals et al., 1996). Pathogen-induced necrosis triggers local resistance (LR; top, infected tissue of the plant) and systemic acquired resistance (SAR; bottom, uninfected tissue of the plant). The DIR1 protein is proposed to act as a co-signal or a translocator for release of the mobile signal into the vascular system and/or chaperone the signal through the plant. Both LR and SAR are blocked in transgenic *NahG* plants expressing salicylate hydroxylase that are unable to accumulate salicylic acid (SA). *SID2* is known to be involved in the synthesis of SA. *dir1-1* and *NahG* but not the *npr1* mutant phenotype can be rescued by treatment of plants with the chemical benzo (1,2,3) thiadiazole-7-cabothionic acid S-methyl ester (BTH), a functional analog of SA. NPR1 is known to act as a key regulator of SAR, interacts with TGA transcription factors and mediates pathogenesis-related gene expression leading to disease resistance.

Genes implicated in the regulation of SAR were identified during the course of screens aimed at recovering different classes of defense response mutants. Some of these involved screening for mutants specifically affected in the ability to express *PR* genes, or display enhanced disease resistance following treatment with SA analogs (Cao et al., 1994; Delaney et al., 1995; Shah et al., 1997). In other cases, mutants originally identified as being compromised in other defense responses (e.g. basal defense) have been subsequently implicated in SAR regulation.

1.7.1. NPR1

Mutations in the *Arabidopsis NON EXPRESSOR OF PR1* (*NPR1*) gene were recovered in multiple genetic screens. The *npr1-1* mutant was isolated in a screen for plants unable to express SA-inducible *PR* genes after exposure to INA (Cao et al. 1994). It was subsequently shown to be compromised in SA-, INA- and avirulent pathogen-induced SAR against *P. syringae* (Cao et al., 1994). The *npr1-2* and *npr1-3* mutants were isolated in a different genetic screen aimed at identifying genes involved in basal resistance against virulent *P. syringae* (Glazebrook et al., 1996). The *npr1-2* mutant was then shown to be defective in INA-induced SAR against *H. parasitica* (Cao et al., 1997). The *npr1-5* mutant, earlier known as *SA insensitive1* (*sai1*), was identified in a different genetic screen for genes involved in SA-induced *PR* gene expression (Shah et al., 1997). The *nim1* mutants (*non-inducible immunity1*) are allelic to *npr1* and were identified during screens for INA-induced disease resistance against *H. parasitica* (Delaney et al., 1995). All the *npr1/nim1* mutants except *nim1-5* carry recessive, loss-of-function mutations and the *npr1* phenotype cannot be rescued by treatment with SA, indicating that NPR1 functions downstream of this metabolite in the SAR signaling pathway (Ryals et al., 1997).

Arabidopsis npr1 mutants are more susceptible to normally virulent strains of *P. syringae* (Cao et al., 1994), *H. parasitica* (Delaney et al., 1995; McDowell et al., 2000; Liu et al., 2005) and *E. cichoracearum* (Xiao et al., 2005). HR-associated cell death is increased in *npr1* mutants after infection with avirulent strains of *P. syringae* (Cao et al., 1994; Vanacker et al., 2001; Zhang et al., 2004). *npr1* mutants are also compromised in resistance against certain avirulent races of *P. syringae* (Shah et al., 1997), *H. parasitica* (Delaney et al., 1995; Liu et al., 2005; McDowell et al., 2000) and *E. cichoracearum*

(Xiao et al., 2005) as well as in ISR (Pieterse et al., 1998) and BABA-IR (Cohen, 2002; Jakab et al., 2001). NPR1 does not appear to be required for signaling through all *R*-genes tested (see for example Rairdan and Delaney, 2002) and conflicting results about the requirement of NPR1 for R-gene signaling have been reported in different studies. Potentiation as a result of priming by BTH of both *Pst*-induced *PAL* expression and wound- or water-infiltration-induced *PAL* activation and callose production is absent in the *npr1* mutant (Conrath et al., 2001 and Kohler et al., 2002). Together, these results implicate NPR1 as a positive regulator of SAR, ISR, BABA-IR, basal defenses, priming and race-specific resistance mediated through some, but not all, *R*-genes.

The *npr1* mutants do not express SA-inducible *PR* genes (e.g. *PR-1*, *PR-2*, *PR-5*) in response to treatment with SA, INA or BTH (Cao et al., 1994; Delaney et al., 1995). However, low levels of *PR* genes are observed after pathogen challenge or in certain double or triple mutants in combination with *npr1* (Dong, 2001; Glazebrook et al., 1996; Liu et al., 2005), indicating the presence of NPR1-independent, SA-dependent disease resistance pathways. Although *npr1* mutants cannot respond to SA, they accumulate high amounts of SA after pathogen challenge (Delaney et al., 1995). Of note, the expression of *SID2* that encodes a key enzyme in SA synthesis (see section 1.6.1.1.) is elevated in *npr1* mutant plants (Wildermuth et al., 2001). Also, the *npr1* seedlings grown in the presence of SA bleach and die after the development of cotyledons (Cao et al., 1997) suggesting that NPR1 may be involved in feedback regulation of SA accumulation and detoxification.

The *NPR1* gene encodes a novel protein containing a nuclear localization sequence (NLS) and two protein-protein interaction domains known as the ankyrin repeat domain (ARD) and the Broad Complex, Tramtrack, and Bric-a-brac/Pox virus and Zinc finger (BTB/POZ) domain (Cao et al. 1997; Sedgwick and Smerdon, 1999). Some *npr1* mutants are affected in conserved amino acids of the ARD suggesting that this domain is important for NPR1 function. NPR1 does not contain any known DNA binding domains suggesting that it may not function as a sequence-specific transcription factor. NPR1 displays sequence similarity with the mammalian transcriptional regulator IκB, and contains several N-terminal lysines and serines that are potentially involved in ubiquitination and phosphorylation events, respectively (Ryals et al., 1997). However,

there is no evidence to date to prove that NPR1 is regulated by either or both of these mechanisms. On the contrary, there is strong evidence to support post-translational regulation of NPR1 by oxido-reduction (redox) changes of conserved cysteines (Fobert and Després, 2005; Mou et al., 2003; see below).

There are several genes encoding proteins related to NPR1 identified in both dicot and monocotyledonous plants including tobacco, tomato, canola, papaya and rice, suggesting that NPR1 is well conserved across the plant kingdom (Chern et al., 2005a; Ekengren et al., 2003; Liu et al., 2002b). The tobacco and tomato NPR1 homologs have been shown to be required for resistance against TMV (Liu et al., 2002b) and *P. syringae avrPto* (Ekengren et al., 2003) indicating that the tobacco and tomato NPR1 homologs are also implicated in disease resistance.

Under uninduced conditions, *NPR1* is expressed constitutively at relatively low levels and after SAR induction by SA treatment or pathogen inoculation, the levels are increased (Cao et al. 1997; Ryals et al., 1997). Mutation of the cognate binding sites for WRKY class transcription factors (i.e. W-boxes, (T)GACC/T), in the promoter of *NPR1* compromises its expression, indicating the involvement of this class of transcription factors in *NPR1* gene regulation (Yu et al., 2001). Interestingly, the expression of various *WRKY* genes is dependent on *NPR1* (Yu et al., 2001) and several genes that are differentially regulated in the *npr1* mutant contain W-boxes in their promoters (Eulgem, 2005; Pan et al., 2004).

Immunoblots with an NPR1 antibody detected the protein in both the cytosolic and nuclear fractions under uninduced conditions (Després et al., 2000). However, under similar conditions, an NPR1-GFP fusion localizes predominantly in the cytoplasm (Kinkema et al., 2000; Mou et al., 2003). It has been proposed that the cytoplasmic NPR1 exists as oligomers held together by intermolecular disulfide bonds (Mou et al., 2003). After treatment with SA, the cellular redox conditions become more reductive (Mou et al., 2003). The observation that mutation of conserved cysteine residues within NPR1 (C82 and C216) leads to its constitutive nuclear localization and the activation of *PR* genes (Mou et al., 2003), led to the proposal that SA-induced redox changes trigger the reduction of NPR1 intermolecular disulfide bonds, resulting in monomerization and its

subsequent translocation to the nucleus (Mou et al., 2003). Nuclear localization of NPR1 also requires the NLS (Kinkema et al., 2000).

Using the yeast-two hybrid system several groups have shown the existence of physical interactions between NPR1 and TGA factors that belong to the bZIP family of transcription factors (Després et al., 2000; Zhang et al., 1999; Zhou et al., 2000). Several TGA factors bind specifically to an SA-responsive element (*LS7*) found in the *Arabidopsis PR-1* promoter, suggesting that NPR1 regulates defense gene expression by interacting with bZIP transcription factors (Després et al., 2000; Zhang et al., 1999; Zhou et al., 2000).

The interaction of NPR1 with two members of the *Arabidopsis* TGA family (TGA1 and TGA4) is regulated by the redox status of two conserved cysteines located in the C-terminal region of these factors (Després et al., 2003). Treatment of cells with SA leads to the reduction of these cysteines and strong interaction with NPR1 (Després et al., 2003). Therefore, translocation of NPR1 to the nucleus and its interaction with TGA factors seem to be regulated post-translationally through redox changes of conserved cysteines (Després et al., 2003; Mou et al., 2003) suggesting that SAR is regulated by redox conditions in the cell.

The interaction of NPR1 with TGA factors has been well studied and shown to play an important role for the functioning of TGA factors in vivo. The NPR1 protein stimulates the DNA binding properties of interacting TGA factors in vitro, including the reduced form of TGA1 (Després et al., 2000 and 2003). Protein extracts from wild-type plants expressing a chimeric TGA2:GAL4 DNA-binding domain (DB) protein were able to bind a probe containing GAL4 binding sites substantially better than extracts prepared from *npr1* plants expressing the chimeric protein (Fan and Dong, 2002). Also, activation of a reporter gene under the control of a promoter containing GAL4 binding sites was detected only in wild-type transgenic plants expressing the TGA2:GAL4 DB fusion, and not in the *npr1* mutant. In a different study, it was shown that TGA2 and TGA3 bind to the *PR-1* promoter only in the presence of SA and a functional NPR1 (Johnson et al., 2003). All these results together indicate that NPR1 and SA are important for both DNA binding and transactivation of TGA factors.

Using yeast two-hybrid screens several NPR1 or NIM1 interacting proteins (NIMINs) have been identified in *Arabidopsis* and rice (Chern et al., 2005a; Weigel et al., 2005). They contain either acidic or basic domains that may serve as effector domains or nuclear localization signals, respectively. The expression of *NIMIN* genes occurs transiently after SA treatment. The overexpression of NIMIN1 in *Arabidopsis* resulted in reduced *PR* gene expression in response to SA treatment or infection with avirulent *P. syringae*. Similar to *npr1* mutants, SAR was compromised against virulent *P. syringae*, but unlike *npr1* mutant plants, the NIMIN1 overexpressors were compromised in *R*-gene mediated resistance against *P. syringae* (*avrRpt2*). Overexpression of a mutant form of NIMIN1 that is unable to interact with NPR1 had no effect on *PR* gene expression or disease resistance, indicating that NIMIN1 function is mediated through NPR1 (Weigel et al., 2005). Also, mutation and RNA interference (RNAi) suppression of *NIMIN1* resulted in increased expression of *PR* genes but had no measurable effects on resistance to *P. syringae*. Therefore, functional analysis of NIMIN1 suggests that it may represent a negative regulator of NPR1.

The overexpression of *NPR1* in *Arabidopsis* plants led to enhanced disease resistance against *P. syringae* and *H. parasitica* (Cao et al., 1998; Friedrich et al., 2001) and *E. cichoracearum* (Friedrich et al., 2001). In one study (Cao et al., 1998), most transgenic lines did not exhibit constitutive expression of *PR* genes. Instead, the increased resistance in these lines was correlated with stronger, rather than faster, *PR* gene expression (Cao et al., 1998). It was also demonstrated that a direct correlation existed between levels of NPR1 protein and disease resistance. In a second study, Friedrich et al. (2001) demonstrated that enhanced resistance was correlated with faster, rather than higher levels of expression of *PR* genes. Also, transgenic plants overexpressing *NPR1* were capable of mounting a defense response against *H. parasitica* when treated with low levels of BTH, ineffective in untransformed plants (Friedrich et al., 2001). Expression of NPR1 in a heterologous host such as tomato resulted in substantial resistance against virulent strains of *P. syringae* and *F. oxysporum*, moderate resistance to *Xanthomonas campestris*, *Ralstonia solanacearum* and *Stemphylium solani*, but no enhanced resistance to *Phytophthora infestans*, Cucumber Mosaic Virus, and Tomato Yellow Leaf Curl Virus (Lin et al., 2004). Levels of resistance to *P. syringae* and *F. oxysporum* were reported to

be comparable, but not as complete, as those conferred by *R*-genes. In most cases, enhanced disease resistance was correlated with increased levels of NPR1. However in some instances, levels of NPR1 did not correlate with enhanced disease resistance leading the authors to propose that resistance may require a threshold level of *NPR1* expression. Also, there was no correlation between the levels of *PR* gene expression and NPR1 levels or disease resistance (Lin et al., 2004). Therefore, it is not quite clear exactly how overexpression of NPR1 leads to enhanced resistance in tomato.

Overexpression of *Arabidopsis NPR1* (Chern et al., 2001) and the rice *NPR1 HOMOLOG 1* (*NH1*), in rice (Chern et al., 2005b), led to enhanced resistance against the bacterial blight-causing pathogen *Xanthomonas oryzae* pv. *oryzae* suggesting that NPR1 function is conserved between monocots and dicots.

It was observed that resistance to *X. oryzae* conferred by NPR1 was not as effective as *R*-gene resistance. However, substantial reduction of pathogen growth was observed in the leaf central vein which limited bacterial spread and enhanced survival of the rice plants. Similar to overexpression studies in tomato, a threshold level of NPR1 was shown to be required for resistance against *X. oryzae*. Overexpression of NH1 in rice also led to the development of spontaneous lesions and accumulation of hydrogen peroxide (Fitzgerald et al., 2004). Neither of these phenomena has been reported in dicotyledonous plants overexpressing NPR1 (Fitzgerald et al., 2004). Unlike untransformed rice plants that contain high levels of SA, transgenic plants overexpressing NH1 displayed low levels of SA suggesting that NPR1 may be involved in the regulation of SA metabolism (Cao et al., 1998; Chern et al., 2005b; Friedrich et al., 2001; Lin et al., 2004).

Sequencing of the *Arabidopsis* genome led to the identification of six *NPR1*-related genes (Liu et al., 2005). The product of *NPR2* (At4g26120) is the closest member that shares 61.3% identity, followed by NPR4 (At4g19660; 36%), NPR3 (At5g45110; 34.5%), NPR6 (At3g57130; 21.4%) and NPR5 (At2g41370; 19.9%). NPR1-NPR4 share sequence similarity throughout the predicted protein, including BTB/POZ and ARD domains. Among all the *NPR1*-related genes, *NPR1* has been very well characterized and recently studies have been reported on *NPR4* (Liu et al. 2005). Similar to NPR1, NPR4 localizes to the nucleus and interacts with the same set of TGA transcription factors. The

NPR4 transcript levels increase rapidly after treatment with SA and remain elevated for a day whereas the levels decrease rapidly after treatment with MeJA, an inducer of the JA pathway (Liu et al., 2005). The disease resistance and *PR* gene expression phenotype in *npr4* mutant is not as strong as *npr1*, suggesting that the role of NPR4 is not as prominent as NPR1.

1.7.2. DIR1

Screens for SAR mutants involving the spraying of chemicals such as INA bypass the initial signal production steps of SAR, since INA was shown to act at the same point as, or even downstream of SA in the SAR signal transduction pathway. Despite reports of a possible role for SA in SAR, and correlation of *PR* gene expression with SAR, virtually no information was available on the molecular mechanisms that result in the induction, transmission and expression of SAR prior to 2002. In an attempt to isolate genes that are upstream of the site of action of SA and involved the SAR signal transduction pathway, Maldonado et al. (2002) screened a population of T-DNA mutagenized *Arabidopsis* for individuals unable to mount SAR following pre-inoculation with an avirulent pathogen. One semi-dominant mutant, called *defective in induced resistance1-1* (*dir1-1*) was identified. *dir1-1* plants are specifically compromised in the SAR pathway; they behave as the wild-type to primary infection with virulent and avirulent pathogens (Maldonado et al., 2002). At the molecular level, expression of the defense genes, *PR-1*, *PR-5* and *GLUTATHIONE-S-TRANSFERASE* (*GST*) was similar in the parental ecotype Wassilewskija (Ws) and *dir1-1* mutants in local leaves following inoculation with avirulent *Pseudomonas syringae* pv. *maculicola* (*Psm*), but greatly reduced in the systemic, uninoculated leaves of *dir1-1* plants. When sprayed with INA and subsequently challenged with virulent bacteria, *dir1-1* plants remained symptomless, indicating that SAR can be effectively induced by SA analogs in this mutant. This demonstrates that the *DIR1* gene functions upstream of SA in the SAR signal transduction pathway (Maldonado et al., 2002). This also suggests that the *dir1-1* defect lies upstream of *NPR1* function in systemic tissues, since the *npr1* mutation is not rescued by INA (Cao et al., 1994).

Cloning of the *DIR1* gene demonstrated that *dir1-1* mutant plants contain a T-DNA insertion in the 3' non-coding region of the affected gene (Maldonado et al., 2002).

This results in a substantial reduction in DIR1 transcript following challenge with *Psm*. Antisense expression of *DIR1* in Ws shows *dir1-1* phenotype, while overexpression of wild-type *DIR1* in the *dir1-1* background rescued the mutant phenotype (Maldonado et al., 2002). Together these results indicate that the *dir1-1* phenotype is specifically due to the loss of *DIR1* function.

DIR1 is predicted to encode a protein containing a hydrophobic amino-terminal signal sequence and the eight cysteine residues that are conserved in all lipid transfer proteins (LTP), suggesting that it is an apoplastic LTP (Maldonado et al., 2002). LTPs are known to bind lipids and transfer them from one membrane to another in vitro (Shah, 2005).

It was speculated that the *dir1-1* phenotype could result in the inability of the infected leaves to produce a systemic signal required for SAR, or uninoculated leaves to perceive such a signal. In an attempt to distinguish between these two possibilities, Maldonado et al. (2002) tested the ability of phloem exudates from infected leaves of Ws and *dir1-1* plants to trigger SAR, as monitored by *PR-1* gene expression, upon infiltration into leaves of both these genotypes. Whereas exudates from *dir1-1* plants infected with avirulent *Psm* were not capable of inducing *PR-1* expression when infiltrated into either Ws or *dir1-1* leaves, *dir1-1* leaves were capable of expressing *PR-1* following infiltration with exudates from Ws leaves. Furthermore, Western blot analysis demonstrated that DIR1 protein was present in exudates from infected Ws leaves, but not in the uninduced Ws or infected *dir1-1* exudates (unpublished results, http://www.science.mcmaster.ca/biology/faculty/cameron/rcamero_research.htm). Together these results argue that DIR1 is required for the production, rather than the perception, of a phloem mobile SAR signal (Maldonado et al., 2002). It was suggested that upon SAR induction, DIR1 may translocate into the phloem where it could act as a co-signal or a translocator for release of the mobile signal into the vascular system and/or chaperone the signal through the plant. This signal may then be perceived in systemic tissues by an unknown SAR signal receptor leading to the induction of SAR (Maldonado et al., 2002).

1.7.3. Other lipid-related SAR regulators

The isolation of DIR1, a putative LTP, as a key player during SAR suggested the potential for lipid-based signaling during this phenomenon. This section reviews

information on other regulators of plant defense responses implicated in lipid metabolism or signaling, even if they may not have been specifically shown to be involved in regulating SAR.

1.7.3.1. EDS1 and PAD4

The enhanced disease susceptibility mutant, *eds1*, was identified in a screen for mutants that were defective in *R*-gene (*RPP1* and *RPP5*) mediated resistance to *H. parasitica* (Parker et al., 1996). Further inspection of *eds1* mutants revealed defects in basal resistance to virulent isolates of *H. parasitica, Erysiphe* spp. and *P. syringae*. The phytoalexin deficient mutant, *pad4*, was identified in a screen for enhanced disease susceptibility to low doses of virulent *Psm* (Glazebrook et al., 1996). Disease analysis revealed that these proteins were usually required by the same spectrum of *Arabidopsis R*-gene mediated resistance involving the TIR-NB-LRR class of proteins and also for SA accumulation (Aarts et al., 1998; Feys et al., 2001; Rustérucci et al., 2001). Both EDS1 and PAD4 were found to contain regions that have homology to eukaryotic lipases (Falk et al., 1999; Jirage et al., 1999) but have not been formally shown to possess lipase activity. In planta, EDS1 exists as molecularly and spatially distinct protein complexes with PAD4 and another structurally-related protein, SENESCENCE-ASSOCIATED GENE101 (SAG101), which is also required for basal and *R*-mediated disease resistance (Feys et al., 2005).

1.7.3.2. SFD1

The *Arabidopsis suppressor of fatty acid desaturase deficiency 1* (*sfd1*) mutation compromised the SAR-conferred enhanced resistance to *Psm* and reduced the SA and *PR-1* gene transcript levels in the distal leaves of plants that were previously exposed to an avirulent pathogen (Nandi et al., 2004). However, the resistance to virulent and avirulent strains of *P. syringae* and the accumulation of elevated levels of SA and *PR-1* gene transcript in the pathogen-inoculated leaves of *sfd1* were not compromised. Treatment of plants with BTH enhanced disease resistance in the *sfd1* mutant plants. *SFD1* encodes a putative dihydroxyacetone phosphate (DHAP) reductase and is capable of complementing DHAP-deficient *Escherichia coli*. SFD1 is required for the synthesis of plastidic glycerolipids, suggesting the involvement of lipids during SAR.

1.7.3.3. SABP2

The SA-binding protein 2 (SABP2) from tobacco has been shown to specifically bind SA with high affinity (Du and Klessig, 1997). Sequence analysis predicted that SABP2 is a lipase belonging to the α/β fold hydrolase super family. The lipase activity of SABP2 has been shown to increase four- to five-fold by addition of SA (Kumar and Klessig, 2003). SABP2 might be a receptor for SA because lipase activity is stimulated by SA binding and this could generate a lipid-derived signal that is important in defence signaling. Also, silencing of SABP2 suppressed local resistance to TMV, induction of *PR-1* gene expression by SA, and development of SAR. These results therefore led the authors to propose that SABP2 is a receptor for SA that is required for the plant immune response. More recently, it was shown that SABP2 has esterase activity with methyl salicylate (MeSA) as the substrate, and that SA was able to inhibit this process (Forouhar et al., 2005). Based on modeling studies of SABP2 with MeSA, it was suggested that SABP2 may be required to convert MeSA to SA as part of the signal transduction pathways that activate SAR and local defense responses (Forouhar et al., 2005). RNAi studies have confirmed that SABP2 is required for SAR against TMV (Kumar et al., 2006).

1.8. Canola and diseases affecting canola

Canola is defined as any of the several varieties of *Brassica* spp. that contain less than 2% erucic acid and whose solid component contains less than 30 micromoles per gram of glucosinolates (Ackman, 1990). *Brassica* plants have been grown in Europe since the 13th century and canola was developed between 1958 and 1974 by two Canadian scientists, Baldur Stefansson and Keith Downey. Canola is the most important source of vegetable oil in the world next to soybean. In 2000–2001, world production of canola totaled 33.86 million tons or 13% of oilseeds produced (Economic Research Service, USDA, 2001). Canola is now a major crop in Canada with a five year average of 11 million acres grown each year. Approximately 70% of vegetable oil products, such as salad oil and margarine, sold in Canada are derived from canola. Canola has an economic impact of $2.5 billion per year in Canada. Canola is also the world's second leading source of protein meal next to soybean.

Brassica crops are among the oldest cultivated plants known to humans. Written records of *Brassicas* are available from ca. 1500 BC (Prakash, 1980) and archaeological evidence of their importance dates back to 5000 BC (Yan, 1990). *Brassica rapa* seems to have had the widest distribution historically. At least 2000 years ago it was distributed from northern Europe to China and Korea, with a majority of diversity occurring in the Himalayan region (Hedge, 1976). *Brassica napus* is believed to have developed in the Mediterranean area where the wild forms of its ancestral species were sympatric; i.e. closely related species occupying the same or overlapping geographic areas without interbreeding. It is possible that *B. napus* arose in cultivation, since no wild forms are known. In addition to *B. napus* and *B. rapa*, *Brassica* includes cultivated species of *B. carinata*, *B. nigra* and *B. oleracea*. The four most widely cultivated species are *B. napus, B. juncea* (Indian mustard), *B. oleracea* (cabbage, kale, broccoli) and *B. rapa* (turnip, Chinese cabbage). All of them are highly polymorphic and include oilseed, root and vegetables crops.

The Triangle of U is a model that describes the relationship and evolution between cultivated species of the genus *Brassica* (Figure 1.2). The "U" triangle was first put forth by Morinaga (1934) and verified later by U (1935). According to this model, there were three different ancestral *Brassica* genomes that combined, in various configurations, to create most of today's common Brassicaceae oilseeds and vegetables. According to this theory, many *Brassica* species were derived from three ancestral genomes, denoted by the letters A, B or C. The letter "n" denotes the haploid number of chromosomes in each genome. Initially, the three species *B. nigra* (2*n*=16, BB; black mustard), *B. oleracea* (2*n*=18, CC), and *B. rapa* (2*n*=20, AA) may have existed as isolated relatives and their interspecific breeding allowed the creation of three new species of tetraploid *Brassicas*; *B. napus* (2*n*=38, AACC), *B. juncea* (2*n*=36, AABB), and *B. carinata* (2*n*=34, BBCC; Ethiopian mustard). Since they are derived from genomes of two different species these species are known as allotetraploids or amphidiploids. Due to the small genome size, rapid life cycle, prolific seed production and availability of mutant stocks, *Arabidopsis* has emerged as one of the best model plants. Canola is very closely related to *Arabidopsis*. Both are dicotyledonous plants belonging to the Brassicaceae family. It has been shown that there is conserved order of genes and gene contents in the

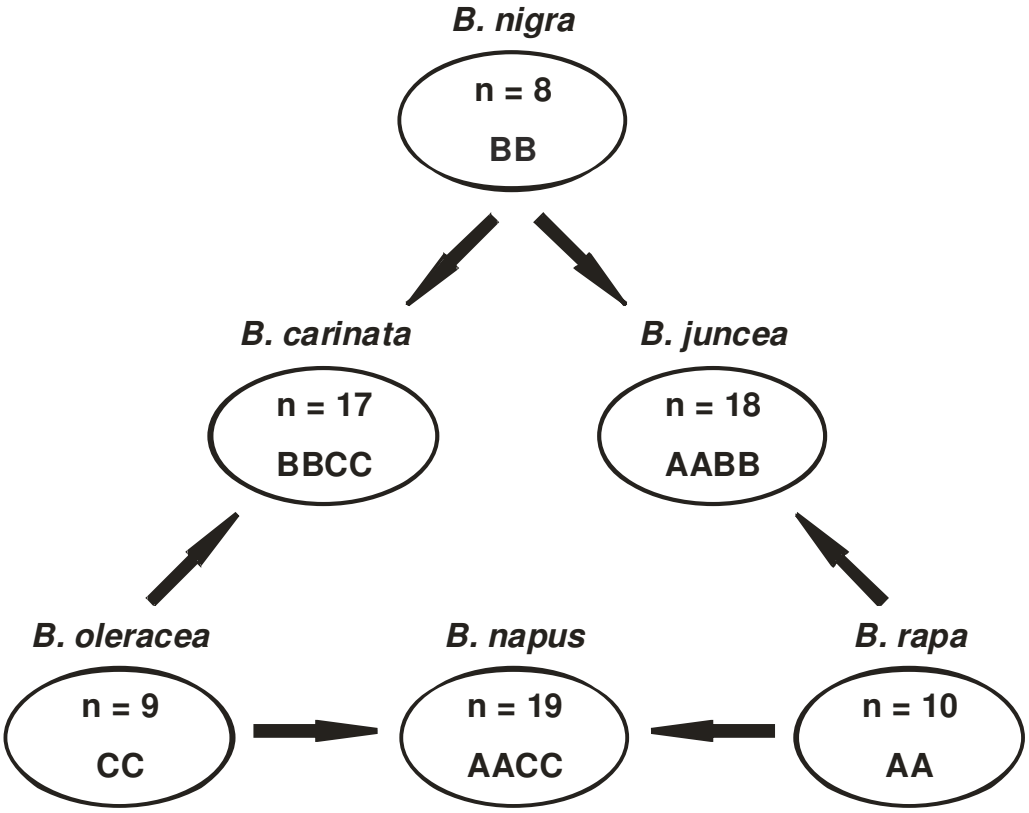


Figure 1.2. The "Triangle of U" representing the genomic relationships among *Brassica* species (U, 1935).

genomes of *Arabidopsis* and canola (Cavell et al., 1998; Scheffler et al., 1997). It has also been reported that there is on average 87% sequence identity between homologous genes from these two species and extensive conservation of molecular markers between large segments of the genomes (Parkin et al., 2005). These genome relationships and the knowledge acquired through the study of *Arabidopsis* can be exploited for isolation and cloning of genes controlling agronomically important traits in canola.

Diseases that affect canola are caused by fungi, oomycetes, bacteria, viruses and phytoplasmas (Table 1.1). Blackleg, caused by a fungal pathogen, *Leptosphaeria maculans* is a major disease affecting canola worldwide and can cause significant yield loss (Howlett et al., 2001; Tewari and Mithen, 1999). Other diseases that have economic impact are damping off caused by *Rhizoctonia solani, Pythium* spp*., Fusarium* spp. and stem rot caused by *Sclerotinia sclerotiorum*. Given the world-wide economic importance of canola, this crop has become an important target for studying disease resistance. To date, studies related to SAR in canola have been very limited (Mahuku et al., 1996).

1.9. Research objectives

It has been clearly demonstrated that plants have the potential to develop SAR towards pathogens after a prior infection with necrosis-causing pathogens or treatment with chemical activators. Significant advances have been made in understanding the genes and the signaling pathways that are involved in regulating disease resistance as well as the chemical signals that modulate the response. Much of the work done on SAR has concentrated on the model plant *Arabidopsis* and some members of the Cucurbitaceae and Solanaceae families (Sticher et al., 1997). However, not much work has been reported related to SAR studies in canola. Therefore, the main goal of my research was to obtain an insight into the molecular mechanisms that are involved in SAR of canola, an economically important member of the Brassicaceae family. In order to achieve this goal, the immediate objective was to characterize SAR in canola using avirulent *Psm* and the chemical BTH in attempts to activate SAR against the virulent pathogens, *Psm* and *L. maculans*. This included studying the associated changes in the plant at the molecular level. The second objective was to study the effect of overexpressing known components of SAR from *Arabidopsis* (NPR1 and DIR1) in canola and test the effectiveness of the resulting transgenic lines against *Psm* and *L. maculans* in combination with SAR pre-

Table 1.1. A list of some of the major diseases affecting *Brassica* spp. (Modified from Tewari and Mithen, 1999).

Disease-Causing Pathogen	**Disease**
Alternaria brassicae	Black spot
Leptosphaeria maculans	Black leg or stem canker
Rhizoctonia solani, Pythium spp., *Fusarium* spp.	Damping off, root rot complex
Albugo candida	Staghead and white rust
Sclerotinia sclerotiorum	Stem rot
Hyaloperonospora parasitica	Downy mildew
Pyrenopeziza brassicae	Light leaf spot
Verticillium dahliae	Verticillium wilt
Plasmodiophora brassicae	Clubroot
Xanthomonas campestris pv. *campestris*	Black rot
Pseudomonas syringae pv. *maculicola*	Pod rot
Xanthomonas campestris pv. *armoraciae*	Leaf spot
Erwinia carotovora	Soft rot
Turnip Crinkle Virus	Crinkle
Cauliflower Mosaic Virus	Mosaic
Phytoplasma	Aster yellows

treatments. The third objective was to isolate putative orthologs of SAR regulators from canola and assess their ability to functionally complement corresponding mutants in *Arabidopsis* as well as enhance disease resistance against *Psm* and *L. maculans* when overexpressed in canola.

CHAPTER 2. Characterization of Systemic Acquired Resistance in *Brassica napus*

Systemic acquired resistance (SAR) is an induced defense response that confers long-lasting protection against a broad range of microbial pathogens. Here it is shown that treatment of *Brassica napus* plants with the SAR inducing chemical BTH significantly enhanced resistance against virulent strains of the bacterial pathogen *Pseudomonas syringae* pv. *maculicola* (*Psm*) and the fungal pathogen *Leptosphaeria maculans*. Localized pre-inoculation of plants with an avirulent strain of *Psm* also enhanced resistance to these pathogens but was not as effective as BTH treatment. Single applications of either SAR-inducing pre-treatment were effective against *Psm*, even when given more than 3 weeks prior to the secondary challenge. The pre-treatments also activated the expression of pathogenesis-related (*PR*) genes, including *B. napus PR-1* (*BnPR-1*) and *BnPR-2*, with higher levels observed in the BTH-treated material. *B. napus* plants expressing a bacterial salicylate hydroxylase transgene (*NahG*) that metabolizes salicylic acid to catechol were substantially compromised in SAR and possessed reduced levels of *PR* gene transcripts when compared to untransformed controls. Thus, SAR in *B. napus* displays many of the hallmarks of classical SAR including long-lasting and broad-host range resistance, association with *PR* gene activation and a requirement for salicylic acid.

2.1. Introduction

Systemic acquired resistance is an induced disease resistance state that is achieved in uninfected parts of a plant following localized exposure to pathogens that cause some form of cell death at the site of infection, such as the hypersensitive response (HR) associated with *Resistance*-gene (*R*-gene) mediated resistance or disease-induced necrosis (Durrant and Dong, 2004; Ryals et al., 1996). SAR has been reported in several dicot and monocot species and is effective against a broad range of viruses, bacteria, oomycetes, and fungi (Kuć, 1982; Sticher et al., 1997). However, SAR is not effective against all

pathogens and the spectrum of resistance varies between plant species (Hammerschmidt and Becker, 1997).

Characteristic features of SAR include the requirement for salicylic acid (SA) and an association with the induction of PR genes and proteins (Durrant and Dong, 2004). Following exposure to pathogens, SA levels increase substantially at the site of infection (locally) and to a lesser extent, in uninfected (systemic) tissues (Ryals et al., 1996; Yalpani et al., 1991). SA accumulation is necessary for SAR, as plants unable to accumulate SA, either through transgenic expression of a bacterial salicylate hydroxylase (*NahG*) gene that metabolizes SA to catechol (Delaney et al., 1994; Gaffney et al., 1993; Lawton et al., 1995), sense suppression of PAL (Pallas et al., 1996), or loss-of-function mutations that prevent SA biosynthesis (Nawrath and Métraux, 1999) are compromised in SAR. Conversely, exogenous application of SA or its functional analogs BTH and INA lead to enhanced resistance to pathogens (Dempsey et al., 1999; Friedrich et al., 1996; Lawton et al., 1996).

Increases in SA levels also trigger the local and systemic expression of a subset of *PR* genes also known as *SAR* genes (Uknes et al., 1992; Ward et al., 1991). The timing of *SAR* gene expression correlates with the onset and duration of SAR (Uknes et al., 1992; Ward et al., 1991), and accumulation of *SAR* genes is compromised in *NahG* plants (Delaney et al., 1994; Lawton et al., 1996). Although the contribution of individual *PR* or *SAR* genes to disease resistance remains unclear (Sticher et al., 1997), their expression provides useful markers for SAR. In particular, *PR-1*, *PR-2* and *PR-5* have been extensively used as markers for the onset of SAR in *Arabidopsis* and tobacco (Durrant and Dong, 2004).

Brassica napus (Linnaeus; canola), is an economically important crop that is grown worldwide and is susceptible to many bacterial and fungal diseases resulting in huge economic losses (Howlett et al., 2001). In particular, blackleg disease caused by the ascomycete *L. maculans* is one of the most serious diseases of oilseed *Brassicas*, including canola grade *B. napus* and *B. rapa.* Nevertheless, SAR has yet to be carefully analyzed in oilseed *Brassicas*. Mahuku et al. (1996) demonstrated that disease lesions caused by a highly virulent strain of *L. maculans* were reduced significantly when adjacent leaves had been previously infected with a weakly virulent strain. Treatment of

B. napus with the SAR-inducing chemical menadione sodium bisulphate (MSB), which appears to induce a form of systemic resistance distinct from SAR, has been shown to enhance resistance to *L. maculans* (Borges et al., 2003) while application of BTH has been shown to be effective at reducing downy mildew caused by *H. parasitica* in *B. oleracea* (Godard et al., 1999; Jensen et al., 1998; Ziadi et al., 2001) and to provide some control of damping-off caused by *Rhizoctonia solani* in *B. napus* (Jensen et al., 1998). BTH treatment of *B. oleracea* seedlings induced β-1,3 glucanase activity and PR-2 protein levels, but had no effect on chitinase activity or the expression of *PR-1*, *PR*-3 and *PR-5* (Ziadi et al., 2001). Of note, no study has assessed the effectiveness of BTH against *L. maculans*, compared the protection conferred by biologically- versus chemically-induced SAR, or tested the requirement of SA for SAR in *B. napus*.

2.2. Materials and methods

2.2.1. Plant growth conditions

Brassica napus (Linnaeus), cultivar (cv.) Westar, plants were grown in the greenhouse at 22ºC with 18-h day light at 190 μE m^{-2} s^{-1}. Seedlings used for fungal disease testing were grown in cabinets at similar temperature and light conditions with 50% relative humidity.

2.2.2. Production of transgenic *Brassica napus* expressing the *NahG* gene

Transgenic *B. napus* (cv. Westar) plants expressing *NahG* gene were generated in Dr. Fobert's lab prior to starting this thesis project. *B. napus* plants were transformed by *Agrobacterium*-mediated transformation according to Tsang et al. (2003). The plasmid used for transformation contained the *NahG* gene driven by the Cauliflower Mosaic Virus (CaMV) 35S promoter described by Liu et al. (2005). Rooted plants were transferred to pots for seed and tested for *NahG* gene expression by northern blot analysis.

2.2.3. Chemical treatment

Ten day old cotyledons (for fungal disease testing), four-week-old plants (for bacterial disease testing) or plants at different age (for time course studies) were sprayed with a freshly made solution of BTH (37.5 μg active ingredient [a.i.] ml^{-1}; 178 μM) containing 0.01% v/v Tween 20. This concentration is similar to that previously found to be effective in *Arabidopsis* (Lawton et al., 1995). Control plants were sprayed with water

containing Tween 20 or received no treatment. BTH was provided as Bion WG50 (50% active ingredient) by the Syngenta Crop Protection Canada, Inc. (Guelph, Canada).

2.2.4. Pathogen inoculations for SAR against *Pseudomonas syringae* and bacterial quantification

Pseudomomas syringae (van Hall) pv. *maculicola* (*Psm*) strains were obtained from Dr John Taylor (Horticulture Research International, Wellesbourne, U.K.). These were grown at 30ºC in 2YT (1.6% tryptone, 1% yeast extract, 0.5% NaCl) medium for 16 h, re-suspended in 10 mM $MgCl_2$ and diluted to 1 X 10^6 and 1 X 10^5 colony forming units (cfu ml^{-1}).

To test the effect of SAR at different times after primary inoculations, starting with 10-day old seedlings, either the cotyledons (for the week 3 time point) or the 1st and 2nd true leaves (all other time points) were infiltrated with avirulent *Psm* 1120B, from the abaxial side, with a 1-ml plastic syringe. For controls, plants were infiltrated with 10 mM $MgCl_2$ alone or received no pre-treatment. Secondary inoculations on 3rd and 4th true leaves were done on 4-week-old plants by infiltrating with virulent *Psm* 1848B. Three days later, 8 leaf discs (4 mm diameter) per plant were collected randomly from 3rd and 4th leaves. Leaf samples were ground in 500 µl of 10 mM $MgCl_2$, serially diluted and spread on *Pseudomonas* Agar-F medium (Difco, Sparks, MD, U.S.A.) plates. These were incubated at 30ºC for 3 days before recording the colony counts. The average cfu per leaf disc (cfu ld^{-1}) were analyzed statistically using an unpaired Student's *t*-test at $p = 0.05$ (Witte, 1989). Each time point represents 9 samples, with each sample consisting of 8 leaf discs.

For monitoring bacterial growth at various times following infection with virulent *Psm* 1848B, the 1st and 2nd leaves of 3-week-old plants were infiltrated with avirulent *Psm* 1120B. Plants of similar age were also sprayed with BTH. Four days later, secondary inoculations were performed with virulent *Psm* 1848B on the 3rd and 4th leaves. Leaf disc samples were collected every day after the secondary infection, up to 5 days. These were processed as described above. For SAR comparison between untransformed and *NahG* plants, secondary inoculations were done 4 days after BTH treatment or primary inoculation with avirulent *Psm* 1120B, before quantifying growth of

virulent *Psm*. Extracts from leaves infiltrated with buffer alone did not yield any colonies at the dilutions used for the infected plants.

2.2.5. Pathogen inoculation for SAR against *Leptosphaeria maculans* and disease assessment

Leptosphaeria maculans (Desm.) Ces. & de Not. [anamorph *Phoma lingam* (Tode ex Fr.) Desm.] strain GL-11, containing the *Escherichia coli uidA* (*GUS*) gene, was obtained from Dr. Ginette Séguin Swartz (Agriculture and Agri-Food Canada, Saskatoon, Canada) and grown on solid V8 juice agar medium supplemented with 0.75% $CaCO_3$, 1.5% agar, 100 µg ml^{-1} streptomycin, 50 µg ml^{-1} hygromycin and 40 µg ml^{-1} Rose bengal in dark light at 24ºC. Pycnidiospore suspensions were prepared according to Mahuku et al. (1996) to a final concentration of 1 X 10^7 spores ml^{-1} in water and stored at –80ºC.

Ten-day-old cotyledons were used for testing SAR against *L. maculans*. Primary inoculations were done on one of the cotyledons by infiltrating with avirulent *Psm* 1120B on the abaxial side at a concentration of 10^6 cfu ml^{-1}. For controls, cotyledons were infiltrated with 10 mM $MgCl_2$ alone. Secondary inoculations were done on cotyledons from seedlings pre-treated with BTH or pre-inoculated with avirulent *Psm* 1120B by placing 10 µl of 1 X 10^7 spore ml^{-1} *L. maculans* suspension onto a wound in their center. The seedlings were misted for the first two days (95% relative humidity) and after 6 more days, they were harvested, frozen in liquid nitrogen and stored at –80ºC.

Histological and fluorometric GUS assays were performed according to Jefferson et al. (1987) using 5-bromo-4-chloro-3-indolyl-ß-D-glucuronic acid cyclohexyl ammonium salt (X-Gluc) and 4- methylumbelliferone ß-D-galactopyranoside (MUG) as substrates, respectively. Protein concentration was quantified by the Bradford assay according to the manufacturer's instructions (Bio-Rad, Hercules, CA, U.S.A.) using bovine serum albumen (BSA) as a standard. Fluorometric GUS assays are presented as pmol methylumbelliferone (MU) µg $protein^{-1}$ min^{-1}. Trypan blue staining of *NahG* cotyledons was performed as previously described (Liu et al., 2005) except that samples were cleared with 70% ethanol. Sections were mounted on a slide and photographed using an Optronics International (Chelmsford, MA, U.S.A.) DEI 750 digital microscope camera.

2.2.6. Salicylic acid analysis

Leaf material (~300 mg) was ground in liquid nitrogen and placed into a 15-ml round bottom glass culture tube containing 3 ml of extraction solvent (90% methanol, 9% water, 1% glacial acetic acid) and 200 ng of internal standard (3, 4, 5, 6-tetradeuterosalicylic acid, CDN Isotopes Inc. Quebec, Canada).

After sonication for 5 min at 30°C in a sonicating water bath, the material was centrifuged for 5 min at 2000 rpm at 10°C. The supernatant was removed and the pellet extracted with another 3 ml of extraction solvent. After centrifugation, the pellet was extracted again, this time with 2 ml of methanol. The supernatants were pooled and the methanol was evaporated under a stream of nitrogen. The remaining aqueous solution was placed on ice, and its pH adjusted to 10 with 0.1N NaOH. The mixture was then extracted with 3ml of dichloromethane. The aqueous layer was transferred to a new tube and the dichloromethane layer was back-extracted with 2 ml of 0.1N NaOH. The pooled aqueous layers were acidified with 5% HCl and extracted 3 times with a 1-ml mixture of ethylacetate:cyclohexane (1:1, v/v). The solvents were removed under a stream of nitrogen and the samples were provided to Mr. Darwin Reed (Plant Natural Products Group, National Research Council, Saskatoon) for further processing and analysis, as described in the following paragraph.

The residue was dissolved in 10 µl of derivitizing reagent (1:1 mixture of N,O-bis(trimethylsilyl)acetamide and pyridine). Gas chromatography/mass spectrometry (GC/MS) analysis was accomplished using an Agilent 6890 GC equipped with an auto injector split 30:1 onto a DB-5MS column (30M X 0.25 mm i.d., J&W Scientific, Folsom, CA, U.S.A.) which was temperature-programmed from 175°C to 300°C at 5°C min^{-1}. The column was connected to an Agilent 5973 mass selective detector. For maximum sensitivity the detector was run under standard conditions with a limited scan range of 265 to 275 m/z which gave 37.9 scans per second. For maximum selectivity and accurate quantitation, ions specific to the compounds of interest were monitored and integrated. For the trimethylsilyl derivative of SA, m/z 267 was used and for the tetradeutero internal standard, m/z 271 was used. The detection limit of the TMS derivative under these conditions was ~ 0.1 ng μl^{-1} and the responses were linear within the concentration range used.

2.2.7. Northern blot analysis

Total RNA was isolated from frozen samples using Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA, U.S.A.). Five µg RNA was denatured and separated on a 1.2% (w/v) agarose 3% (v/v) formaldehyde gel, photographed under UV light to record amounts loaded and blotted to nylon membrane (GeneScreen Plus Hybridization Transfer Membrane; NEN Life Sciences Products, Inc., Boston, MA, U.S.A.). Membranes were cross-linked with UV light, hybridized at 65ºC in QuickHyb solution (Stratagene) with probes radiolabelled with (α- ^{32}P)dCTP using random primers (Invitrogen, Burlington, Ontario). Two hours after hybridization, membranes were washed twice at room temperature in 2X SSC, 1% SDS for 15 min and once in 0.1X SSC and 0.1% SDS at 65 ºC for 30 min and later exposed onto a X-ray film (Sterlin Diagnostics, Newark, DE, U.S.A.) at –80ºC. For re-hybridization with different genes, membranes were stripped by boiling for 30 min in 0.1 X SSC and 0.1% SDS. Hybridization probes contained the entire coding regions of *Brassica napus PR-1* (U70666, herein referred to as *BnPR-1*) and *Brassica PR-2* (X77990, *BnPR-2*) genes previously shown to be induced following pathogen challenge (Fristensky et al., 1999; Newman et al., 1994) and BTH (Hennin et al., 2001). Because I was not aware of any characterized *Brassica PR-5*–related genes expressed under these conditions, a related gene originally isolated from flower buds was chosen for analysis (U71244, 5). Coding regions were isolated by reverse transcriptase polymerase chain reaction (PCR) amplification of mRNA isolated from *B. napus* seedlings treated with 2 mM SA (Sigma, St. Louis, MO, U.S.A.) for 18 h using the following oligonucleotide primers: *BnPR-1* (5'-ATGAAAGTCACTAACTGTTCTCGAC-3' and 5'-GCCAGTAAACTAGGTAACGGATAA-3'); *BnPR-2* (5'-GGATGTTAGCATCATCACCAATGTTGCTG-3' and 5'-GGAGATTAGTTAAACTTAACACCATATTTAAGCTG-3'); *BnPR-5* (5'-CAATGGCTTCACGAAACCTCTTCAACTTCG-3' and 5'-GTGATTTTAACGGCGATGGTGAGGGCAAAA-3'). Isolated fragments were ligated into the cloning vector pTOPO4 (Stratagene, La Jolla, CA, U.S.A.) and verified by sequencing.

2.3. Results

2.3.1. SAR is effective at reducing disease caused by *Pseudomonas syringae* pv. *maculicola*

In *Arabidopsis*, SAR against *P. syringae* is very effective and has been characterized extensively (Cameron et al., 1994; Cameron et al., 1999; Uknes et al., 1992; Uknes et al., 1993). Accordingly, we initiated our studies of SAR in *B. napus* using this bacterial pathogen. As a first step, a collection of *Psm* strains (obtained from Dr. John Taylor), were infiltrated into leaves of *B. napus* (cv. Westar) to identify those capable of inducing disease symptoms, which could be used to monitor the effectiveness of SAR treatments. At the same time, these strains were assessed for their capacity to elicit an HR, and thus having the potential to trigger SAR (Figure 2.1A). One representative strain for each of the two categories (*Psm* 1848B and *Psm* 1120B, respectively) was chosen for further study.

The effectiveness of pre-inoculation with an avirulent (necrogenic) pathogen at reducing disease (herein referred to as biological SAR), was tested by infiltrating the 1st and 2nd true leaves of plants with 10^6 cfu ml^{-1} of *Psm* 1120B and subsequently infiltrating the 3rd and 4th leaves with 10^5 cfu ml^{-1} of virulent *Psm* 1848B. To assess the effectiveness of BTH at reducing disease (i.e. chemical SAR), a separate group of plants was sprayed with 37.5 μg of a.i. ml^{-1} BTH prior to infiltration with *Psm* 1848B. These plants were grown in parallel and under the same conditions as those used for the biological SAR experiments, and BTH treatments were scheduled to coincide with pre-inoculation treatments. Disease was assessed by visually monitoring symptoms and quantifying viable bacteria in the 3rd and 4th (systemic) leaves. Preliminary experiments indicated that pre-inoculation with 10 mM $MgCl_2$ or spraying with a Tween 20 solution had no significant effect on growth of *Psm* 1848B (data not shown). Accordingly, control plants in most experiments did not receive any pre-treatments. Preliminary trials suggested that reduction of disease symptoms was greatest when *Psm* 1848B was inoculated four days after primary treatments (see below). Therefore, these conditions were selected to quantify bacterial growth at different times following infiltration with *Psm* 1848B.

In leaves from control plants, bacteria multiplied to 4 X 10^4 cfu ld^{-1} after one day and increased to 1.6 X 10^6 cfu ld^{-1} after 4 days before tapering off (Figure 2.1B). Leaves

A

B

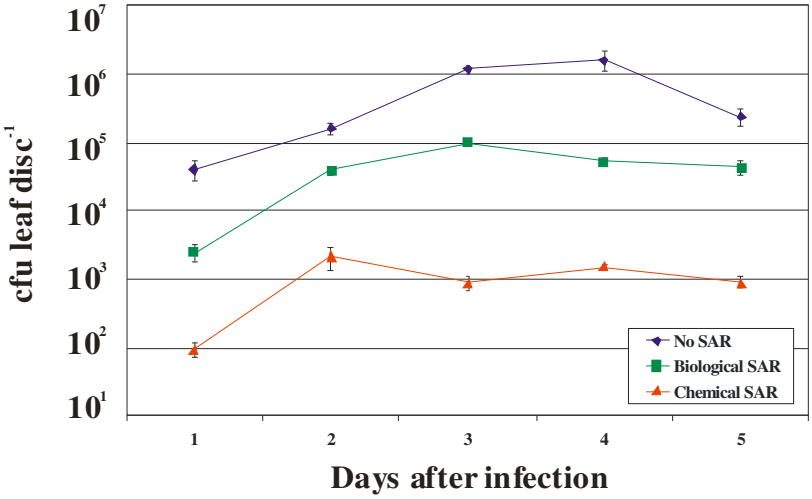


Figure 2.1A. *Brassica napus* leaves showing necrotic symptoms following inoculation with *Pseudomonas syringae* pv. *maculicola* strain 1120B. Left, leaf inoculated with $MgCl_2$ showing no symptoms and right, leaf 48 hours after inoculation with *Psm* strain 1120B (1 X 10^6 cfu ml^{-1}) showing typical necrotic lesions. **2.1B.** *In planta* quantification of *Pseudomonas syringae* pv. *maculicola* strain 1848B growth in *Brassica napus* leaves at different times following SAR-inducing pre-treatments. Control plants (No SAR) did not receive any pre-treatment. Biological SAR consisted of pre-inoculation with avirulent *P. syringae* 1120B (1 X 10^6 cfu ml^{-1}) while the chemical SAR treatment consisted of spraying with BTH (37.5 µg a.i. ml^{-1}). Secondary inoculations with the virulent *P. syringae* strain 1848B (1 X 10^5 cfu ml^{-1}) were performed 4 days after primary treatments. Bacterial counts (cfu leaf $disc^{-1}$) were determined for each treatment at the indicated times after secondary inoculation. Each sample consisted of 8 leaf discs and every data point represents the mean ± SE of 9 samples. This experiment was repeated once with similar results.

from plants that had been pre-inoculated with the avirulent pathogen displayed a 4.1- (day 2 and day 5) to 31-fold (day 4) reduction in bacterial titre when compared to control plants sampled at the same time. Leaves from plants treated with BTH showed a dramatic reduction of bacterial growth (75- to 1328-fold) when compared to control plants. In both the biological and chemical SAR treatments, growth reduction was already apparent one day following infiltration, and was found to be statistically significant at $p \leq 0.05$ using Student's *t*-test. Furthermore, growth reduction measured in the chemical SAR treatment was statistically different from the biological SAR treatment. Leaves from the biological SAR treatment displayed a marked reduction of visible disease symptoms while those treated with BTH did not display any disease symptoms (data not shown).

2.3.2. SAR against *Pseudomonas syringae* pv. *maculicola* is long lasting

To determine the longevity of SAR, bacterial titres were quantified in leaves infected with virulent *Psm* 1848B at various times after pre-treatment with avirulent *Psm* 1120B or BTH. In order to make comparisons between treatments more meaningful, the secondary inoculations were all performed at the same time, on plants of the same age, with the pre-treatments having been performed at different times, accordingly. As shown in Figure 2.2A, pre-inoculation with avirulent *Psm* 1120B resulted in 14- to 19-fold reduction in bacterial growth. Values from all of the time points were statistically different from those obtained in the control plants ($p \leq 0.05$), indicating that biological SAR is effective even when the secondary inoculation was performed 3 weeks after the pre-treatment, the longest time difference tested in this study.

Treatment with BTH resulted in 95- to 1077-fold reduction in bacterial growth with the maximum reduction observed when the secondary inoculation was performed four to seven days after the primary treatment (Figure 2.2A). All values measured in leaves treated with BTH were statistically lower than the control ($p \leq 0.05$). These results indicate that both biological and chemical SAR provide long lasting protection against virulent *Psm* in *B. napus* and confirm the data presented in Figure 1B that treatment with BTH is more effective than pre-inoculation with the avirulent pathogen *Psm* 1120B under the experimental conditions used.

A

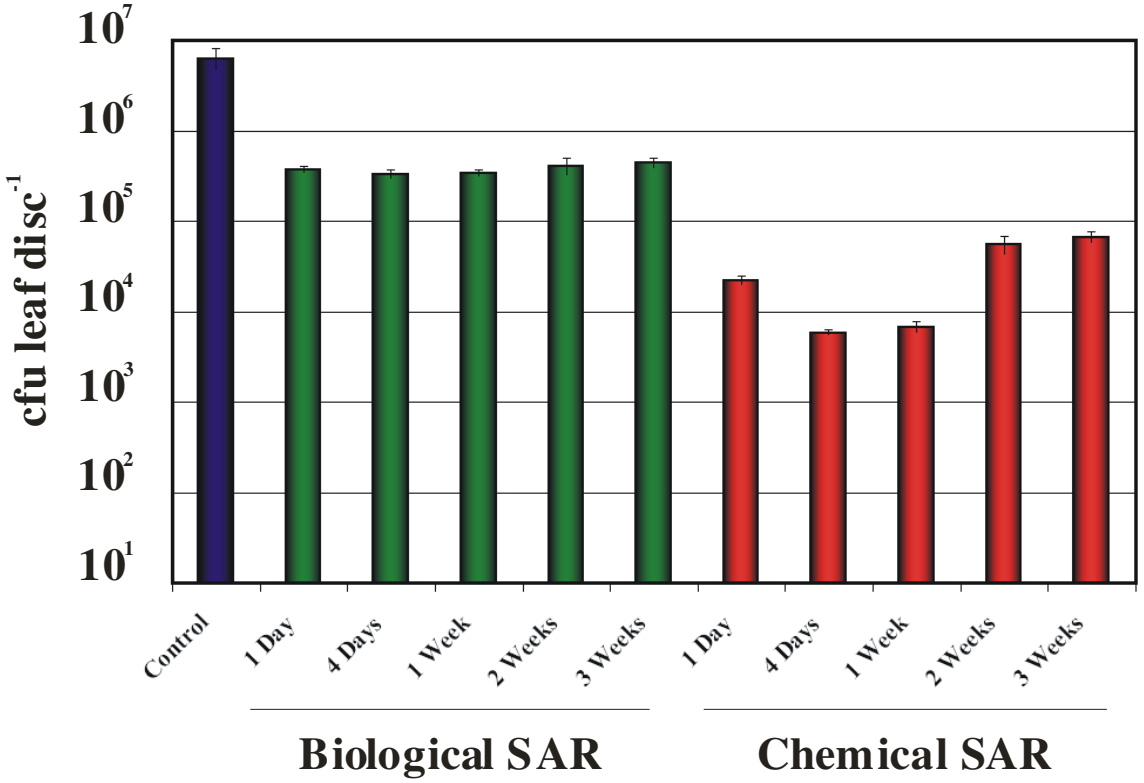


Figure 2.2A. SAR response in wild-type *Brassica napus* plants induced by *Pseudomonas syringae* pv. *maculicola* 1120B or BTH. Plants were either pre-inoculated with a 1 X 10^6 cfu ml^{-1} suspension of avirulent *P. syringae* 1120B (biological SAR) or sprayed with 37.5 µg a.i. ml^{-1} BTH (chemical SAR) for the amount of time indicated before challenge inoculation with the virulent *P. syringae* strain 1848B (1 X 10^5 cfu ml^{-1}). Control plants did not receive any pre-treatments. Three days later, 8 leaf discs per sample were collected and bacterial titres quantified. Each data point represents the mean ± SE of 9 samples. This experiment was repeated 10 times with similar results.

B

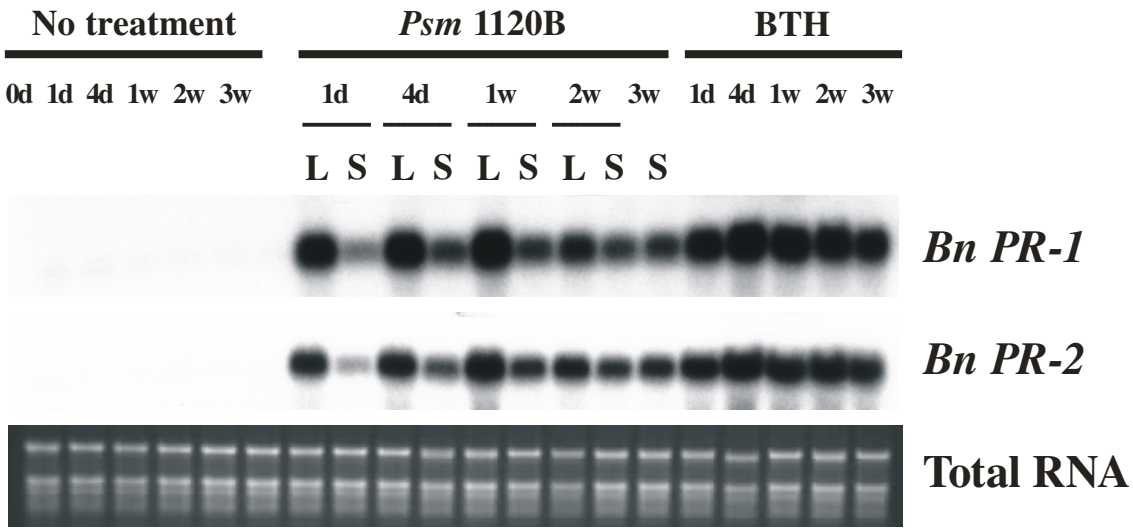


Figure 2.2B. *BnPR* gene transcript accumulation in wild-type *Brassica napus* plants following SAR induction. RNA gel-blot analyses of *BnPR-1* and *BnPR-2* expression at different times following either pre-inoculation with a 1 X 10^6 cfu ml^{-1} suspension of avirulent *P. syringae* 1120B or spraying with 37.5 µg a.i. ml^{-1} BTH for the amount of time indicated. L, local leaf; S, systemic leaf. Five µg of total RNA was loaded for each sample and probed with radioactive labeled DNA probes. The same filters were used for *BnPR-1* and *BnPR-2*. These were stripped of hybridizing DNA between each round of hybridization. Photos of ethidium bromide-stained gels are included as a measure of RNA loading. This experiment was repeated once with similar results.

C

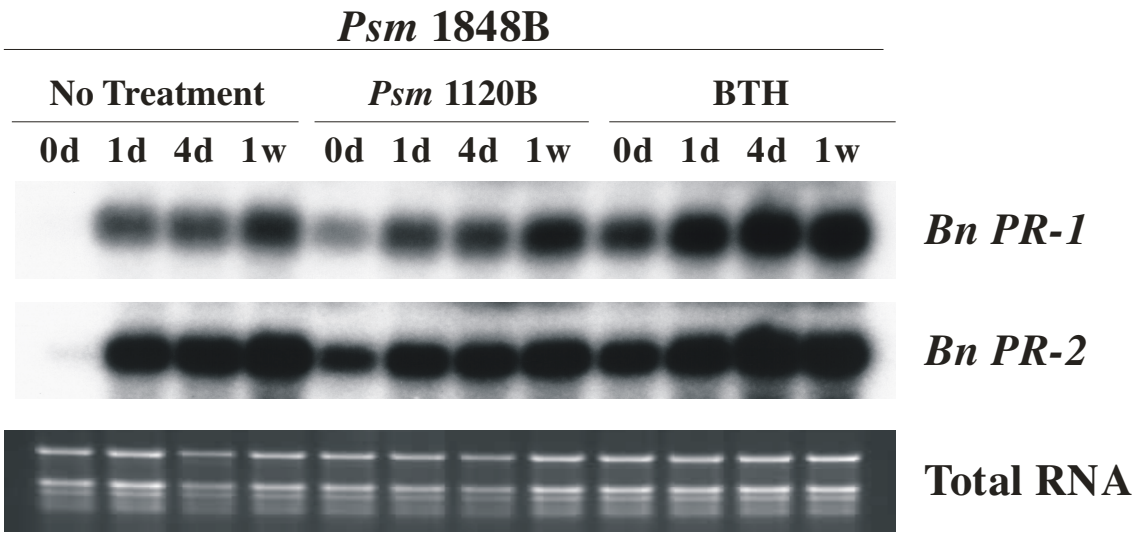


Figure 2.2C. *BnPR* gene transcript accumulation in wild-type *Brassica napus* plants following SAR induction and subsequent challenge with virulent *Pseudomonas syringae* pv. *maculicola* 1848B. RNA gel-blot analyses of *BnPR-1* and *BnPR-2* expression in systemic leaves at different times following challenge inoculation with the virulent *P. syringae* strain 1848B (1 X 10^5 cfu ml^{-1}). Plants had previously received SAR-inducing pre-treatments for 4 days as described in Figure 2.2B. Five µg of total RNA was loaded for each sample and probed with radioactive labeled DNA probes. The same filters were used for *BnPR-1* and *BnPR-2.* These were stripped of hybridizing DNA between each round of hybridization. Photos of ethidium bromide-stained gels are included as a measure of RNA loading. This experiment was repeated once with similar results.

2.3.3. SAR against *Pseudomonas syringae* pv. *maculicola* is correlated with the accumulation of *PR* gene transcripts

Changes in *PR* gene expression associated with SAR induction were analyzed by northern blot hybridization in leaf samples collected at various times following pre-treatments (Figure 2.2B). In leaves inoculated with avirulent *Psm* 1120B (local or L), high levels of *BnPR-1* and *BnPR-2* RNA were already detected 1 day post-infection (Figure 2.2B), the shortest time interval used between primary and secondary inoculations in this study. Steady-state levels of *PR* genes increased slightly during the first week before declining starting at week two. In the uninoculated, systemic (S) leaves, levels of the above gene transcripts were initially substantially lower than observed in infected (L) leaves and gradually increased during the first week (Figure 2.2B). Levels then remained relatively constant and similar to those detected in infected leaves for the remainder of the experiment. Treatment with BTH induced *BnPR-1* and *BnPR-2* transcript accumulation to levels comparable with the highest levels observed in leaves infected with avirulent *Psm* 1120B (Figure 2.2B). Expression of *BnPR-5* was not detected in any of the tissues analyzed (data not shown), while transcripts from none of the *PR* genes analyzed were detected in leaves from untreated plants at any of the developmental stages considered (Figure 2.2B).

PR transcripts were also monitored in leaves at different times following infection with the virulent *Psm* strain 1848B (Figure 2.2C). In plants that had not received SAR pre-treatments, infection with *Psm* 1848B was sufficient to induce *BnPR-1* and *BnPR-2* expression as early as 1 day post-infection. In plants where 1st and 2nd leaves had been inoculated 4 days previously with avirulent *Psm* 1120B, 3rd and 4th leaves infected with *Psm* 1848B expressed slightly higher levels of *BnPR-1* transcripts than observed in plants that did not receive the pre-treatment, but appeared to have similar levels of *BnPR-2*, except at day 0, where *BnPR-1* and *BnPR-2* transcripts were detected only in leaves from plants previously inoculated with the avirulent bacterium. Transcript levels of both of these *PR* genes were higher in BTH-treated plants than those receiving no pre-treatment or pre-inoculated with *Psm* 1120B. Expression of *BnPR-5* was not detected in any of the leaves infected with *Psm* 1848B (data not shown). Together, these results indicate that SAR against *Psm* in mature *B. napus* plants is associated with the induction of *PR* genes

at both the induction and manifestation stages, and that there is a positive relationship between the effectiveness of SAR and the steady-state level of *PR* transcripts.

2.3.4. SAR is effective against *Leptosphaeria maculans*

Studies have shown that early infections of *L. maculans* cause the greatest damage, with infections before the six-leaf stage causing severe yield losses (Howlett et al., 2001). Stems are most susceptible to infection when the plants are in cotyledon or one- to two-leaf stage. Therefore, we tested the effectiveness of SAR against *L. maculans* in *B. napus* plants at the cotyledonary stage.

To trigger biological SAR, one cotyledon from ten-day old seedlings was infiltrated with avirulent *Psm* 1120B (10^6 cfu ml^{-1}) while chemical SAR was induced by spraying seedlings with 37.5 µg a.i. ml^{-1} BTH. Four days later two 10-µl drops of *L. maculans* spore suspension (1 X 10^7 ml^{-1}) were placed on the cotyledons. In the biological SAR treatment, this secondary infection was performed on the cotyledon that did not receive the pre-inoculation. The *L. maculans* strain used in this study (GL-11) expressed the *E. coli uidA* (*GUS*) gene under the control of the CaMV35S promoter. Accordingly, qualitative and quantitative analysis of GUS activity was exploited as a measure of fungal growth.

Histological staining for GUS activity 8 and 13 days after *L. maculans* infection revealed that pre-inoculation of one of the cotyledons with avirulent *Psm* 1120B substantially decreased the area of fungal growth (Figure 2.3A). This reduction was even more dramatic in cotyledons treated with BTH. Quantitatively, GUS activity was reduced by 2.3-fold in seedlings pre-inoculated with avirulent *Psm* 1120B and 77-fold following treatment with BTH (Figure 2.3B). These differences were statistically significant ($p \leq 0.05$) and indicate that SAR induced at the cotyledon stage of *B. napus* plants is effective against *L. maculans*.

BnPR-1 and *BnPR-2* transcripts accumulated to high levels in cotyledons infected with avirulent *Psm* 1120B and following BTH treatment (Figure 2.4A, "L" samples). Expression of *PR* genes was much lower in the systemic (S), uninfected cotyledon. In the absence of pre-treatment, infection with *L. maculans* induced low level expression of *BnPR-1* and *BnPR-2* (Figure 2.4B). Pre-inoculation with avirulent *Psm* 1120B resulted in higher levels of *PR* gene transcripts in cotyledons infected with *L. maculans*, while

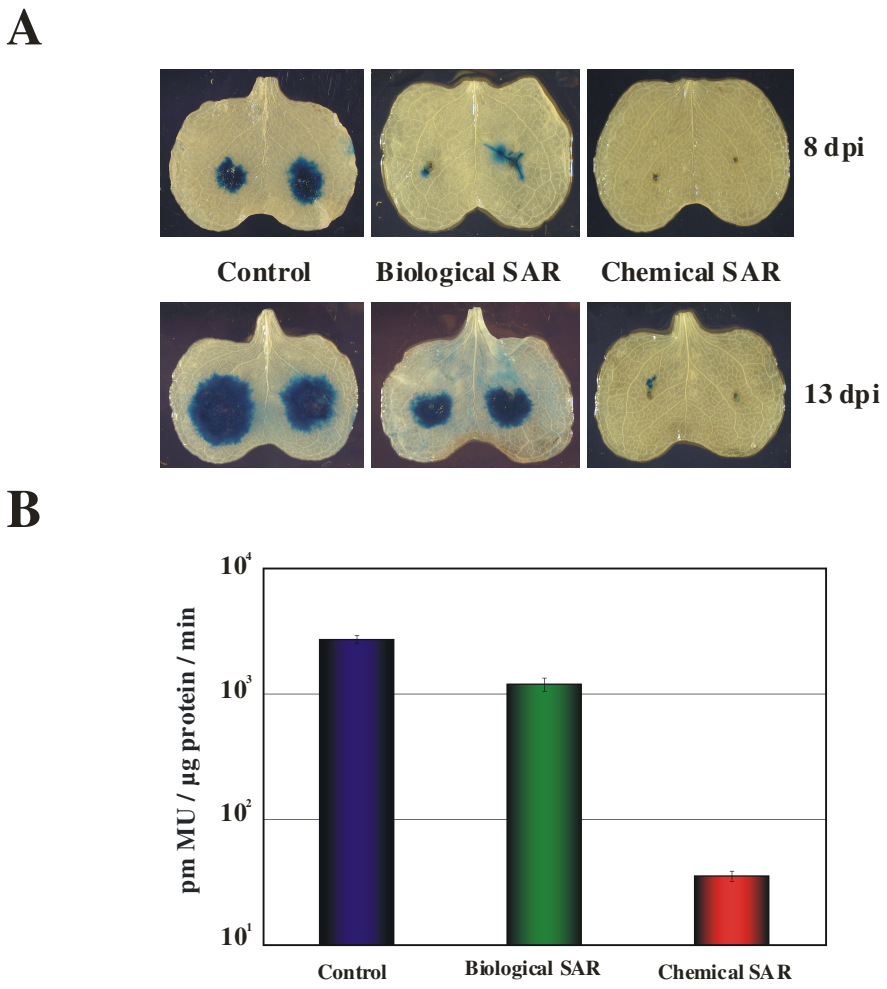


Figure 2.3. Effectiveness of SAR-inducing pre-treatments in wild-type *Brassica napus* cotyledons against *Leptosphaeria maculans*. Six-day old *B. napus* seedlings were either sprayed with water (control), pre-inoculated with 10^6 cfu ml^{-1} of avirluent *P. syringae* 1120B (biological SAR) or sprayed with 37.5 µg a.i. ml^{-1} BTH (chemical SAR). Four days later, 10 µL drops of *L. maculans* spores (1 X 10^7 cfu ml^{-1}) were placed on cotyledons and incubated for 8 or 13 days. In the biological SAR pre-treatment, different cotyledons received the primary and secondary inoculations. **A.** Histological staining of *L. maculans* fungal hyphae for GUS expression using X-Gluc. Hyphal growth 8 or 13 dpi of the fungus is indicated by blue coloration; **B.** Quantitative GUS expression analyses in 8 dpi cotyledons using MUG as substrate. Reduction in growth of *L. maculans* is indicated by decreased GUS expression. Each bar represents the mean ± SE of 6 samples. These experiments were repeated three 3 times with similar results.

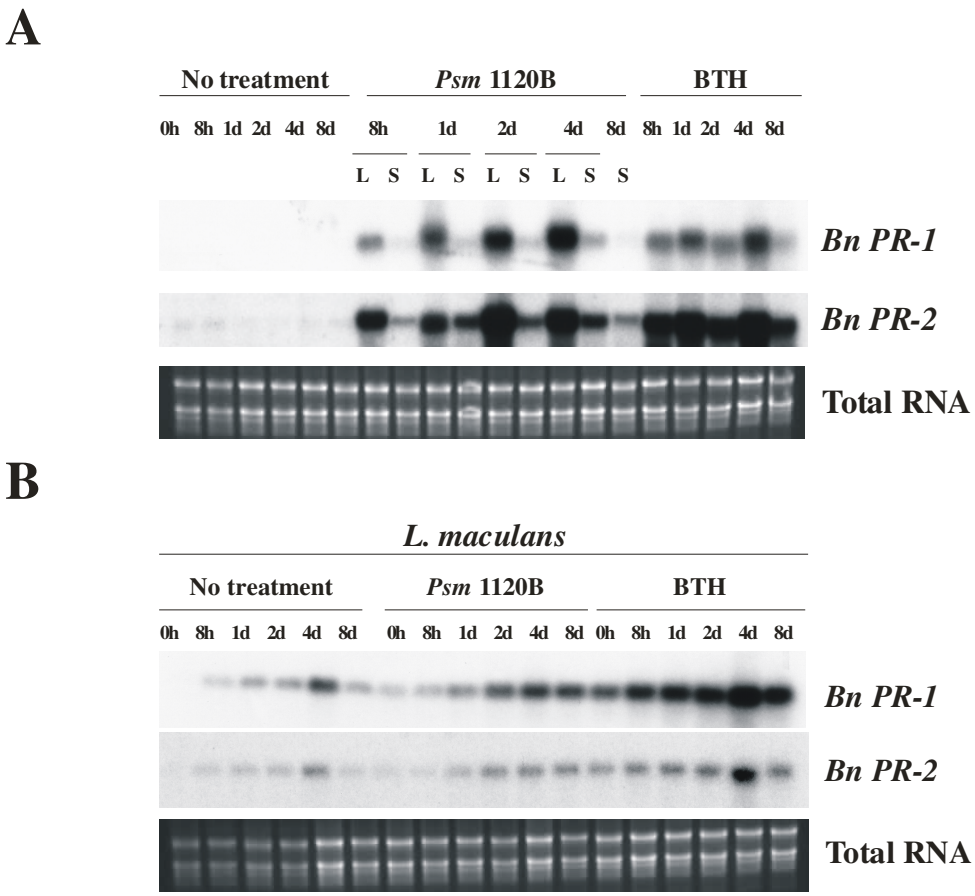


Figure 2.4. *BnPR* gene expression in wild-type *Brassica napus* cotyledons following SAR-inducing pre-treatments. **A.** RNA gel-blot analyses of *BnPR-1* and *BnPR-2* at different times following pre-treatments. *Psm* 1120B indicates pre-inoculation with avirulent *P. syringae* 1120B (1 X 10^6 cfu ml^{-1}); BTH indicates seedlings were sprayed with 37.5 µg a.i. ml^{-1} BTH. L, local cotyledon; S, systemic cotyledon. **B.** RNA gel-blot analyses of *BnPR-1* and *BnPR-2* at different times in cotyledons following challenge inoculation with *L. maculans* (1 X 10^7 cfu ml^{-1}) spores. Cotyledons had previously received the pre-treatments for 4 days as described in **A**. Separate cotyledons of the same seedling were used for seedlings that received both bacterial and fungal inoculations. Five µg of total RNA was loaded for each sample and probed with radioactive labeled DNA probes. The same filters were used for *BnPR-1* and *BnPR-2* and stripped of hybridizing DNA between each round of hybridization. Photos of ethidium bromide-stained gels are included as a measure of RNA loading. These experiments were repeated once with similar results.

spraying with BTH induced the highest levels of *PR* gene expression in these tissues. Thus, as observed in mature plants infected with virulent *Psm* 1848B, there is a positive correlation between the effectiveness of SAR against *L. maculans* and the expression of *PR* genes in *B. napus* seedlings.

2.3.5. Systemic acquired resistance is compromised in *NahG Brassica napus* plants

The requirement of SA for SAR in *B. napus* was tested in transgenic plants expressing the bacterial salicylate hydroxylase gene (*NahG*; Figure 2.5A) that converts SA to catechol (Gaffney et al., 1993). To confirm the efficacy of the *NahG* transgene, levels of free SA were measured in local and systemic leaves of plants 4 days after pre-inoculation with avirulent *Psm* 1120B. As shown in Figure 2.5B, pre-inoculation of untransformed, wild-type plants with this pathogen resulted in a 28-fold increase in SA levels in local tissues and a roughly 2-fold increase in systemic leaves. In contrast, the *NahG* plants did not show any substantial increase in free SA levels in either locally infected or systemic leaves (Figure 2.5B).

In the absence of SAR inductive pre-treatments, bacterial titres measured in leaves of *NahG* plants infected with virulent *Psm* 1848 were 2-fold higher than those measured in untransformed plants (Figure 2.5C). This difference was statistically different ($p \leq 0.05$). To determine if SAR could be induced in *NahG* plants, the 3rd and 4th leaves of untransformed and *NahG* plants were infiltrated with the virulent *Psm* 1848B four days after pre-inoculation of 1st and 2nd leaves with the avirulent *Psm* 1120B, and bacterial growth quantified as described above. After pre-treatment with *Psm* 1120B, untransformed plants showed a 4-fold reduction in the growth of *Psm* 1848B whereas the *NahG* plants did not show any significant decrease (Figure 2.5C). *BnPR-1* and *BnPR-2* transcripts were observed in local and systemic leaves of *NahG* plants 4 days after pre-inoculation with avirulent *Psm* 1120B (Figure 2.5D). However, levels of expression in systemic leaves were reduced compared to those observed in untransformed plants. This was most apparent with *BnPR-2*.

The binary T-DNA plasmid used to generate the *NahG* plants possessed a chimeric selectable marker containing the *GUS* gene (Polowick et al., 2000). Accordingly, we were unable to use this reporter to measure growth of *L. maculan*s. Instead, fungal growth was assessed by monitoring mycelial mass using trypan blue

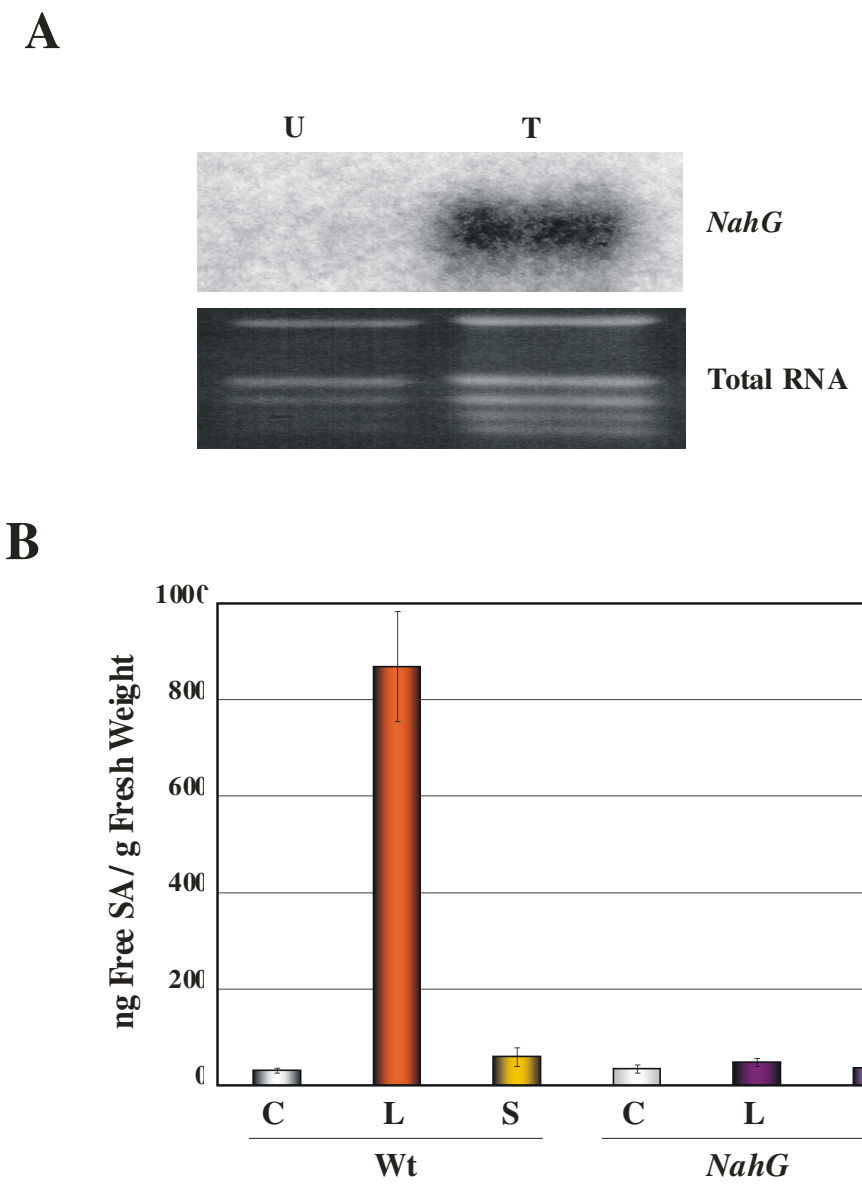


Figure 2.5A. Northern blot showing expression of the *NahG* gene in transgenic *Brassica napus* plants. Five µg of total RNA was loaded for each sample (U, untransformed and T, transgenic *NahG* plants) and probed with radioactively labeled *NahG* probe. A photo of the ethidium bromide-stained gel is included as a measure of RNA loading. **2.5B.** Free salicylic acid in wild-type and *NahG* plants. Untreated control leaves (C), local (L) and systemic (S) leaf tissues, four days after infiltration with 1 X 10^6 cfu ml^{-1} of avirulent *P. syringae* 1120B were analyzed for free salicylic acid using GC/MS analysis. Each bar represents the mean ± SE of 3 samples.

C

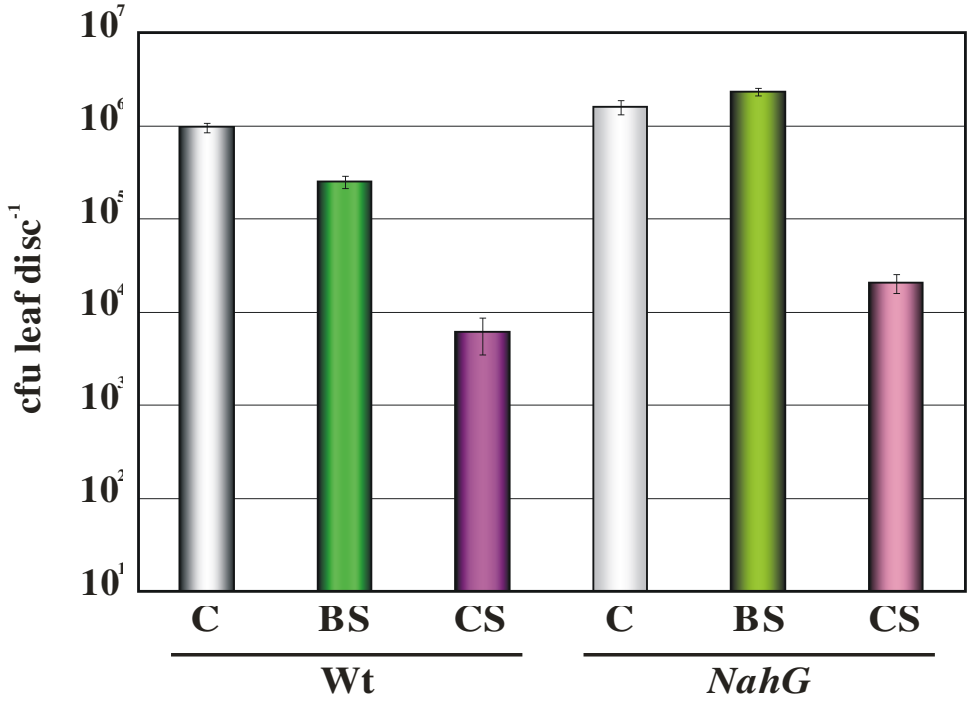


Figure 2.5C. SAR response against *Pseudomonas syringae* pv. *maculicola* in *NahG* *Brassica napus* plants. First and second leaves of three-week-old wild-type (open bars) or *NahG* transgenic (hatched bars) *B. napus* plants were infiltrated with 10 mM $MgCl_2$ (control; C) or pre-inoculated with 1 X 10^6 cfu ml^{-1} of avirulent *P. syringae* 1120B (biological SAR; BS). Alternatively, plants were sprayed with 37.5 µg a.i. ml^{-1} BTH (chemical SAR; CS). Four days later the 3rd and 4th leaves of each plant were challenge inoculated with the virulent *P. syringae* strain 1848B. Three days after the second inoculation, eight leaf discs were collected randomly from 3rd and 4th leaves and bacterial titres quantified. Each data point represents the mean ± SE of 9 samples. This experiment was repeated twice with similar results.

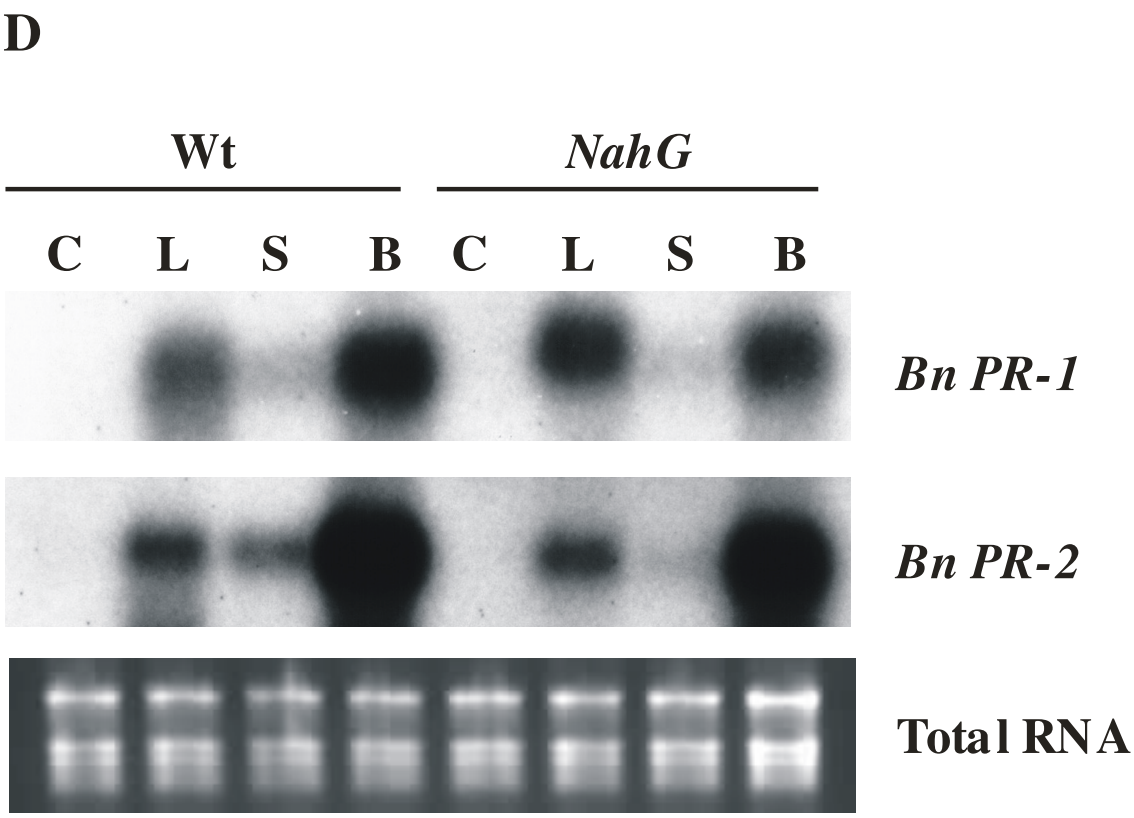


Figure 2.5D. RNA gel-blot analyses *BnPR1* and *BnPR2* expression in wild-type and *NahG Brassica napus* plants at day 4 after SAR pre-treatment. C, untreated control; L, local (1st and 2nd) leaf pre-inoculated with 1 X 10^6 cfu ml^{-1} avirulent *P. syringae* 1120B; S, systemic (3rd and 4th) leaf of the same plants; B, plant sprayed with 37.5 µg a.i. ml^{-1} BTH. Five µg of total RNA was loaded for each sample and probed with radioactively labeled DNA probes. The same filters were used for *BnPR-1* and *BnPR-2.* These were stripped of hybridizing DNA between each round of hybridization. A photo of the ethidium bromide-stained gel is included as a measure of RNA loading. This experiment was repeated once with similar results.

staining. Regardless of whether *NahG* seedlings were pre-inoculated with avirulent *Psm* 1120B, the fungus grew to entirely cover the cotyledons (Figure 2.6A bottom row). By comparison, fungal growth was not as extensive on wild-type cotyledons and pre-inoculation was modestly effective at reducing fungal growth (Figure 2.6A, top row). Thus, expression of *NahG* at the seedling stage compromised SAR against *L. maculans*.

BnPR-1 and *BnPR-2* transcripts continued to be expressed in local and systemic cotyledons of *NahG* seedlings after pre-inoculation with avirulent *Psm* 1120B (Figure 2.6B) as well as in cotyledons infected with *L. maculans* (Figure 2.6C). However, comparison of expression profiles with those observed in untransformed material indicated reduced levels of *PR* gene transcripts in cotyledons inoculated with avirulent *Psm* 1120B one and four days post-inoculation (compare Figure 2.4A and 2.6B). Furthermore, levels of *PR* genes in *NahG* cotyledons infected with *L. maculans* were no higher in seedlings pretreated with avirulent *Psm* 1120B than in the controls (Figure 2.6C), whereas a clear difference was observed between these two treatments in untransformed cotyledons (Figure 2.4B). Therefore, the loss of SAR observed in the *NahG* seedlings is correlated with reduced expression of *BnPR-1* and *BnPR-2*.

In *Arabidopsis* and tobacco, loss of SAR observed in *NahG* plants can be rescued by treatment with BTH (Friedrich et al., 1996; Lawton et al., 1996). This chemical is thought to act at the same point as, or downstream of, SA in the signaling pathway leading to SAR (Friedrich et al., 1996; Lawton et al., 1996). To test whether the loss of resistance in *NahG B. napus* was specifically attributed to interference with SA signaling, plants and seedlings were treated with BTH and assessed for disease resistance. Following BTH treatment, titres of virulent *Psm* 1848B in leaves of *NahG* plants were significantly lower (77-fold) than those measured in the absence of pre-treatment or following pre-inoculation with avirulent *P. syringae* 1120B (Figure 2.5C). These differences were statistically significant ($p \leq 0.05$). However, titres in *NahG* plants treated with BTH continued to be statistically higher ($p \leq 0.05$) than those measured in leaves of untransformed plants treated with BTH. Treatment of *NahG* plants with BTH also induced relatively high levels of *BnPR-1* and *BnPR-2* transcripts (Figure 2.5D).

Trypan blue staining of *NahG* cotyledons after BTH treatment indicated that growth of *L. maculans* was significantly reduced when compared to cotyledons that did

A

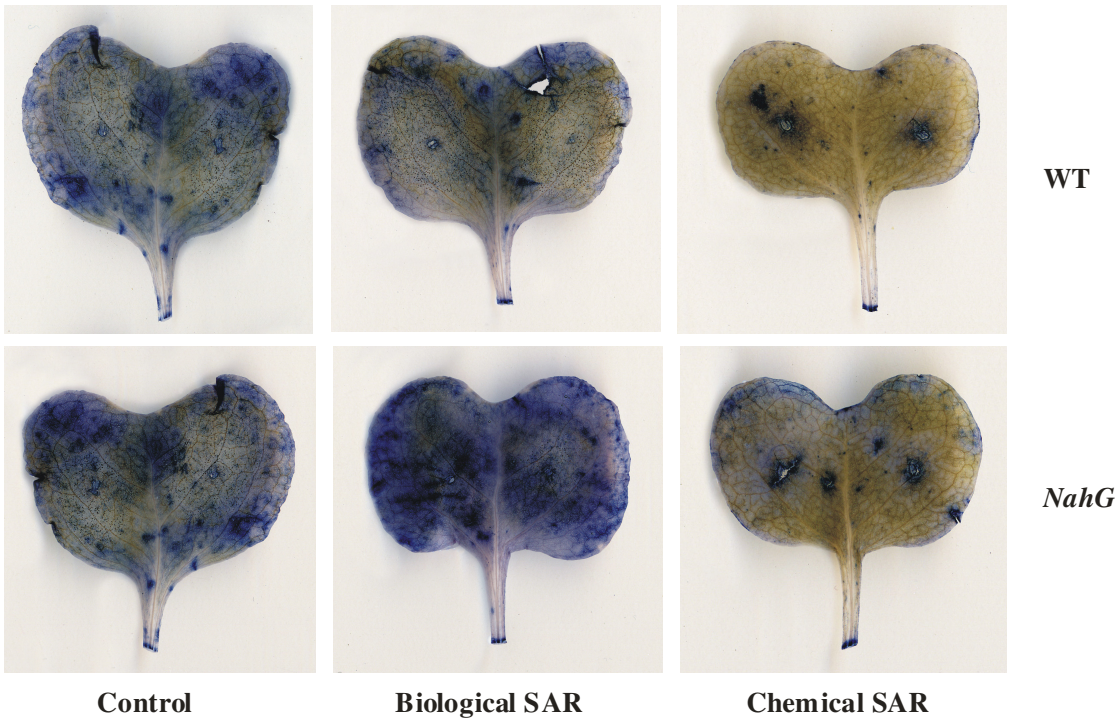


Figure 2.6A. SAR response against *Leptosphaeria maculans* in *NahG Brassica napus* cotyledons. Trypan blue staining of cotyledons from wild-type and *NahG* seedlings 13 days after infection with *L. maculans*. Control, no pretreatment; Biological SAR, other cotyledon of the seedling was infiltrated with 1 X 10^6 cfu ml^{-1} of avirulent *P. syringae* 1120B 4 days before infection with *L. maculans*; Chemical SAR, seedlings were sprayed with 37.5 µg a.i. ml^{-1} BTH 4 days before infection with *L. maculans*. Hyphal growth is indicated by blue coloration. This experiment was repeated three times with similar results.

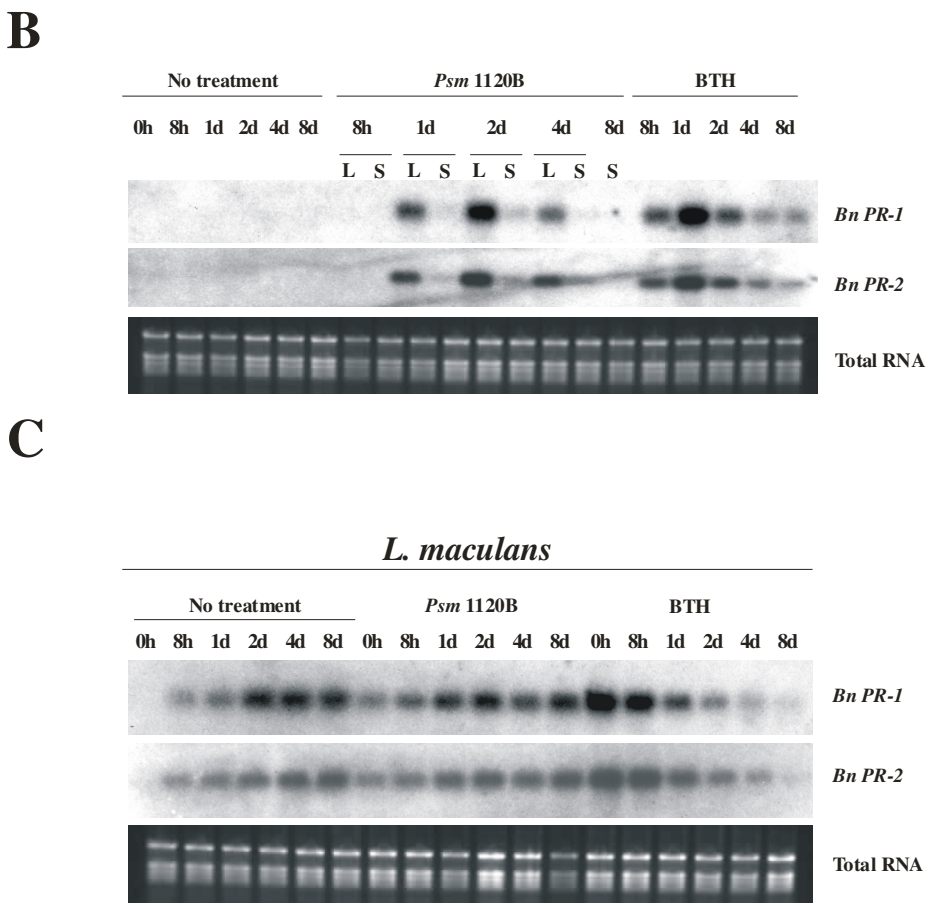


Figure 2.6B and C. *BnPR* gene expression in *NahG Brassica napus* cotyledons. B. RNA gel-blot analyses of *BnPR-1* and *BnPR-2* expression in *NahG B. napus* cotyledons at different times following pre-treatment with 1 X 10^6 cfu ml^{-1} of avirulent *P. syringae* (*Psm*1120B) or 37.5 µg a.i. ml^{-1} BTH (BTH). L, local cotyledon; S, systemic cotyledon. C. RNA gel-blot analyses of the induction of *BnPR-1* and *BnPR-2* in *NahG B. napus* cotyledons at different times following secondary inoculation with virulent *L. maculans* (1 X 10^7 cfu ml^{-1}) spores. Cotyledons had previously received the pre-treatments for 4 days as described in B. Separate cotyledons of the same seedling were used for seedlings that received both bacterial and fungal inoculations. In both B and C, five µg of total RNA was loaded for each sample and probed with radioactive labeled DNA probes. The same filters were used for *BnPR-1* and *BnPR-2.* These were stripped of hybridizing DNA between each round of hybridization. Photos of ethidium bromide-stained gels are included as a measure of RNA loading. These experiments were repeated once with similar results.

not receive any pre-treatment (Figure 2.6A). Together, these data indicate that the SA signaling pathway is critical for effective SAR in *B. napus*.

2.4. Discussion

Results presented in this Chapter demonstrate that localized pre-inoculation with an avirulent pathogen and application of the chemical BTH effectively enhances resistance of *B. napus* plants to virulent strains of the bacterial pathogen *P. syringae* pv. *maculicola* and the fungal pathogen *L. maculans*. Importantly, enhanced resistance to both pathogens displays the hallmarks of a typical SAR response, including the accumulation of *PR* gene transcripts and a requirement for SA.

Pre-inoculation with an avirulent pathogen and BTH treatment of *B. napus* plants resulted in the rapid accumulation of *PR-1* and *PR-2* transcripts; two genes widely accepted as markers for SAR (Ryals et al., 1996). Comparisons of different treatments indicate that the expression levels of these marker genes correlated with the effectiveness of SAR. For example, plants pre-inoculated with avirulent *Psm* 1120B expressed slightly higher levels of *PR* genes in systemic leaves compared to untreated plants and displayed only moderately higher levels of resistance, while plants treated with BTH expressed the highest levels of *PR* gene mRNA and the most resistance (Figures 2.2-2.4). Furthermore, pre-inoculation of *NahG* plants with *Psm* 1120B failed to elicit a SAR response and did not induce the accumulation of *PR* gene transcripts in the systemic leaves (Figures 2.5, 2.6). This correlation of *BnPR* gene expression with SAR suggests that the proteins they encode could contribute to the observed increase in disease resistance.

Hennin et al. (2001) also reported rapid induction of *PR-1* and *PR-2* transcripts in *B. napus* following treatment with BTH. However, these authors did not correlate *PR* gene expression with disease resistance. BTH treatment also led to the enhancement of β-1,3-glucanase (PR-2) enzymatic activity and protein levels in *B. oleracea* seedlings, before and after infection with *H. parasitica* (Ziadi et al., 2001). However, whereas our study and that of Hennin et al. (2001) clearly observed rapid and dramatic increases in *BnPR-1* mRNA, Ziadi et al. (2001) detected only weak and slow induction of *PR-1*. One possible explanation for this discrepancy is that the *BnPR-1* cDNA used as a probe encodes a protein distinct from the one recognized by the PR-1 antibody, which was raised against a tobacco isoform. Alternatively, the differences may be attributed to post-

transcriptional regulation of *PR-1* or to the higher levels of BTH used in the present study and that of Hennin et al. (2001). Ziadi et al. (2001) also failed to detect substantial changes in the expression of PR-5 protein, which is consistent with the inability to detect *PR-5* mRNA following pathogen challenge or during SAR in the present study. Together, these results suggest that expression of *BnPR-5* may not be associated with SAR in *Brassica* species.

The conclusion that SAR in *B. napus* requires SA is based on two lines of evidence. First, BTH, a structural analog of SA, was highly effective at enhancing resistance to *P. syringae* and *L. maculans*. In other plant species, BTH and SA induce the expression of the same set of *SAR* genes and confer resistance to the same spectrum of pathogens (Friedrich et al., 1996; Lawton et al., 1996). Both compounds are thought to activate SAR through the same signaling pathway, possibly by binding to the same receptor(s) (Ryals et al., 1996). Second, blocking the pathogen-induced accumulation of free SA by expression of the *NahG* transgene resulted in plants compromised in biological SAR. However, *NahG B. napus* plants mounted an effective SAR in response to BTH treatment (Figures 2.5, 2.6), indicating that loss of SAR following pre-inoculation with the avirulent pathogen was largely due to the inability to accumulate SA.

In addition to being unable to mount an effective SAR response, *Arabidopsis* and tobacco *NahG* plants have been reported to be severely compromised in basal resistance (Delaney et al., 1994). In contrast, *NahG B. napus* plants were only marginally more susceptible to *P. syringae* in the absence of SAR-inducing pre-treatments (Figure 2.5C), suggesting that loss of SA preferentially compromises SAR, but not basal resistance, against this pathogen in *B. napus*. Also, *B. napus NahG* plants appear to express substantially more residual *PR* gene mRNA in response to challenge by an avirulent pathogen than similar *Arabidopsis* plants (Delaney et al., 1994; Kus et al., 2002; Lawton et al., 1996). Analysis by GC-MS confirmed the effective block of SA accumulation in *B. napus NahG* plants (Figure 2.5B). This suggests an important contribution of one or more SA-independent signaling pathways for the pathogen-induced expression of *PR-1* and *PR-2* in this species. Further analysis will be required to test this hypothesis and define the pathways.

The fact that pre-inoculation with an avirulent strain of *P. syringae* and BTH treatment induced the expression of *BnPR-1* and *BnPR-2* and conferred resistance to virulent strains of both *P. syringae* and *L. maculans* suggests that they may activate the same signaling pathways in *B. napus*. This phenomenon was also observed in *Arabidopsis*, tobacco and several other plant species (Uknes et al., 1992, Ward et al., 1991). However, the same is not true of the chemical MSB, which induced SAR against *L. maculans* without activation of *BnPR-1* (Borges et al., 2003). Despite the possibility that they may induce the same signaling cascades, results presented in this Chapter clearly show that the two pre-treatments are not equally effective at conferring SAR. Previous reports suggest that SAR requires an induction time of about 2 days to 2 weeks to be fully established (Sticher et al., 1997). Altering the length of time between the pre-inoculation and the infection with virulent *P. syringae* did not increase the effectiveness of this pretreatment relative to BTH application (Figure 2.2A).

Localized infection with a microbe capable of eliciting plant cell necrosis is thought to trigger the production of a systemic SAR signal which is thought to be mobilized through the phloem (Durrant and Dong, 2004; Maldonado et al., 2002). The nature of the systemic signal is still unknown, although recent evidence suggests the involvement of a lipid-based molecule (Durrant and Dong, 2004; Kumar and Klessig, 2003; Maldonado et al., 2002). Application of the SA analog INA can rescue SAR in the *Arabidopsis dir1-1* mutant, which is unable to produce the systemic signal (Maldonado et al., 2002; also see Chapter 3 section 3.3), indicating that treatment with INA, and therefore probably BTH as well, likely bypasses the requirement for the systemic signal. Accordingly, the relatively low level of SAR triggered by pre-inoculation with the avirulent pathogen, compared to BTH treatment, may indicate that the production, mobilization or perception of the systemic signal is limiting the effectiveness of SAR.. In addition to altering the time between pre-inoculation and the infection with virulent *P. syringae*, the effect of altering the strain of avirulent bacterium and the area of tissue pre-inoculated was also tested, but neither substantially increased SAR (data not shown). It is unlikely that the superior performance of BTH is due to direct growth inhibition of the virulent pathogens, since neither BTH nor its metabolites appear to have antifungal activities (Friedrich et al., 1996). Furthermore, localized infiltration of BTH on

uninfected leaves was equally effective as spraying the entire plant in eliciting SAR (data not shown).

A previous report had demonstrated that pre- or co-infection with a weakly virulent strain of *L. maculans* was effective at inducing SAR against a highly virulent strain in mature *B. napus* plants (Mahuku et al., 1996). My results confirm that pre-infection with an avirulent pathogen is effective at inducing SAR against virulent *L. maculans*. Furthermore, they extend previous findings by demonstrating that the enhanced resistance is associated with *PR* gene induction and requires SA, and that BTH is highly effective at reducing disease caused by *L. maculans*. Importantly, results presented in this Chapter show that SAR against *L. maculans* is effective at the seedling stage, a time at which disease caused by this fungus can be particularly devastating.

CHAPTER 3. Characterization of DIR1 Function in *Brassica napus*

Genetic screens for *Arabidopsis* mutants compromised in SAR have identified several regulators of this phenomenon. The *defective in induced resistance1-1* (*dir1-1*) mutant has been shown to be compromised in the production or movement of a systemic SAR signal. The *DIR1* gene encodes a putative lipid transfer protein (LTP), implicating lipid signaling in SAR. To study DIR1 function in *Brassica napus*, I isolated a *DIR1*-related cDNA from this species and tested its ability to complement the *Arabidopsis dir1-1* mutation. I also overexpressed the *Brassica* gene in wild-type *Arabidopsis* and assessed the consequences on disease resistance. Finally, both the *Brassica* and *Arabidopsis DIR1* cDNAs were overexpressed in *B. napus* and the consequences on disease resistance were assessed.

3.1. Introduction

Plants are constantly exposed to a large number of pathogens resulting in the activation of various defense responses (Lamb et al., 1989; Lamb, 1994). SAR is one such induced disease resistance response that is achieved after the pre-inoculation with pathogens that cause necrosis or local cell death (Ryals, 1996). Early events in SAR signal transduction involve the accumulation of salicylic acid (SA) and the activation of pathogenesis-related (PR) genes and proteins at the site of infection. Ultimately a signal, the nature of which is unknown, is produced and transmitted systemically, probably through the phloem (Jennes and Kuć, 1979). It is unlikely that the signal is SA itself, and recent evidence implicates a putative lipid-based signal (Durrant and Dong, 2004; Shah, 2005). Following perception of the systemic signal, uninfected parts of the plant respond by accumulating SA and activating PR genes and proteins. They also become “primed” to activate various defense responses much more effectively upon exposure to a second, normally virulent pathogen and accordingly display enhanced resistance (Conrath et al., 2002). Exogenous application of SA or the SA analogs, INA and BTH, can also lead to enhanced resistance to disease, referred to as chemical SAR (Friedrich et al., 1996). SAR

has been described in different mono- and dicotyledonous plants including crop plant species such as rice, wheat and canola (Chern et al., 2005; Görlach et al., 1996; Sticher et al., 1997; also see Chapter 1.6.1 and results presented in Chapter 2). However, SAR is not effective against all pathogens and the spectrum of resistance varies between plant species (Hammerschmidt and Becker, 1997).

Genetic screens have been used by various groups to identify regulators of SAR in *Arabidopsis* (Durrant and Dong, 2004; Glazebrook, 2001; Ryals et al., 1996). Several studies identified mutants affected in chemical SAR by pre-treating plants with INA (Cao et al., 1994; Delaney et al., 1995). Included in this collection are multiple recessive, loss-of-function mutant alleles at the *NPR1* (*NON-EXPRESSOR OF PR GENES1*) locus (reviewed in Durrant and Dong, 2004). The *npr1* mutants fail to mount effective chemical SAR or express *PR-1* in response to SA or SA analogues. Furthermore, they are compromised in their ability to mount SAR following pre-inoculation with an avirulent pathogen. Given that its mutant phenotype cannot be rescued by SA, NPR1 likely acts downstream of this metabolite in the signaling pathway (Cao et al., 1997; Delaney et al., 1995; Ryals et al., 1997). This notion is further supported by the observations that *npr1* mutants accumulate higher levels of SA compared to the wild-type following infection with avirulent *Pseudomonas syringae* (Delaney et al., 1995). Thus, NPR1 is unlikely to participate in the production of the systemic SAR signal. NPR1, as well as other regulators of defense, such as *PAD4* (*PHYTOALEXIN DEFICIENT4*; Jirage et al., 1999; Zhou et al., 1998), and *EDS1* (*ENHANCED DISEASE SUSCEPTIBILITY1*; Falk et al., 1999), have also all been implicated in regulating SA-mediated defense responses other than SAR (Aarts et al., 1998; Cao et al., 1994; Delaney et al., 1995; Glazebrook et al., 1997; Feys and Parker, 2000; Feys et al., 2001), and are therefore not specifically associated with this induced defense response.

In an attempt to identify genes acting throughout the SAR pathways, and in particular those potentially acting upstream of the site of SA action, Maldonado et al. (2002) screened a population of T-DNA-tagged *Arabidopsis* for individuals impaired in their ability to mount SAR following localized pre-inoculation with an avirulent pathogen. A single locus was thus identified and called *DEFECTIVE IN INDUCED RESISTANCE1* (*DIR1*). The *dir1-1* mutant plants are specifically impaired in SAR; they

behave like the wild-type in response to local inoculation with avirulent or virulent *P. syringae*. The *dir1-1* phenotype can be fully rescued by exogenous INA indicating that it acts upstream of SA in the signaling pathway (Maldonado et al., 2002). Furthermore, the SA levels were similar in wild-type and *dir1-1* plants both in local and systemic leaves indicating that DIR1 is not required for SA accumulation.

To further explore the requirements for DIR1 in the production of the SAR systemic signal, Maldonado et al. (2002) collected phloem exudates from *dir1-1* and wild-type plants that had been inoculated with an avirulent strain of *P. syringae* and infiltrated them into leaves of *dir1-1* or wild-type plants. Their results revealed that the exudates from wild-type plants induced *PR-1* expression in wild-type and *dir1-1* leaves, while exudates from *dir1-1* leaves or from wild-type plants mock inoculated with buffer had little activity. This suggests that *dir1-1* plants are able to perceive the SAR signal emanating from infected leaves but are unable to produce that signal or mobilize the signal into the phloem. Thus, DIR1 could potentially be a signal for SAR induction or be involved in the production of such a signal (Maldonado et al., 2002). Consistent with this notion, *PR-1* is expressed locally, in infected leaves, but not systemically in the *dir1-1* mutant.

The *DIR1* gene encodes a LTP containing a putative signal peptide at its N-terminal (Maldonado et al., 2002). LTPs belong to a class of plant antimicrobial peptides that also include thionins and defensins (Castro and Fontes, 2005). They are small, basic proteins with eight cysteine residues at conserved positions (Kader, 1997). Positively charged amino acids are exposed on the surface of the protein that is hydrophilic and the hydrophobic residues line the internal cavity, able to accommodate fatty acids, a characteristic structure that is conserved in all LTPs. Many LTPs have been shown to transfer phospholipids between membranes *in vitro* (Zachowski et al., 1998). Sequence analyses identified signal peptides in some LTPs and it has been shown that the signal peptide drives insertion into the endoplasmic reticulum lumen (Madrid, 1991). All non-specific plant LTPs characterized so far contain a signal peptide, and immunolocalization data indicate that they locate to the plant cell wall (Thoma et al., 1993; Garcia-Olmedo et al., 1995). However, besides all these similarities, LTPs are known to be highly divergent in their sequences and function. In *Arabidopsis*, for example, 71 putative LTPs with

highly divergent sequences have been identified (Beisson et al., 2003). They are known to be involved in cutin biosynthesis (Sterk et al., 1991), surface wax formation (Pyee et al., 1994), adaptation to environmental changes (Keresztessy and Hughes, 1998; Soufleri et al., 1996), pathogen defense (Garcia-Olmedo et al., 1995; Jung et al., 2003), pollination (Park and Lord, 2003) and germination (Edqvist and Farbos, 2002). Lipid transfer proteins from barley and maize are known to exhibit antimicrobial activity (Douliez et al., 2000). Transgenic tobacco and *Arabidopsis* plants genetically engineered to express a barley LTP were shown to exhibit enhanced tolerance against pathogens (Molina and Garcia-Olmedo, 1997). It has also been demonstrated that a non-specific LTP from mung bean exhibited antibacterial and antifungal activities (Wang et al., 2004).

It has been proposed that disease occurs not due to the lack of genetic information required to deploy a resistance response, but rather as a result of the inability of the plant to express the information soon enough and with sufficient magnitude to combat the pathogen (Kuć, 1982). Accordingly, numerous studies have attempted to enhance resistance to disease by generating transgenic plants that constitutively express genes implicated in defense responses. Although over expression of individual or pairs of specific *PR* genes has been shown to be effective in some cases (Alexander et al., 1993; Broglie et al., 1991; Liu et al., 1994; Zhu et al., 1994), the protection provided was less effective and much narrower than that rendered by fully-fledged SAR (Lin et al., 2004). Therefore, over expression of key regulators of SAR may be able to produce a stronger defense response by activating several defense-related genes involved in the SAR pathway, resulting in effective resistance against pathogens. For example, constitutive expression of *NPR1* in *Arabidopsis* enhanced resistance against bacterial and oomycete pathogens (Cao et al., 1998; Friedrich et al., 2001). Over expression of *NPR1* in crop plant species such as rice and tomato resulted in enhanced resistance to *Xanthomonas oryzae* pv. *oryzae* and to a spectrum of bacterial and fungal pathogens, respectively (Chern et al. 2001; Lin et al. 2004).

3.2. Materials and Methods

3.2.1. Plant material and growth conditions

Brassica napus (Linnaeus) plants (cv Westar) were grown in the greenhouse at 22ºC with 18-h day light at 190 µE m^{-2} s^{-1}. Seedlings used for fungal disease testing were

grown in cabinets at similar temperature and light conditions with 50% relative humidity.

Seeds of *Arabidopsis thaliana* (L.) Heynh ecotype Wassilewskija (Ws) were obtained from Dr. Roger Rimmer (Agriculture and Agri-Food Canada, Saskatoon Research Centre) and seeds of the *dir1-1* mutant (in Ws background) (Maldonado et al., 2002) were obtained from Dr. Richard Dixon (The Noble Foundation, Ardmore, Oklahoma). *Arabidopsis* plants were grown under different conditions, as described below, depending on the purpose of the experiments.

For selecting transformed *Arabidopsis* plants (transformation procedure is described in section 3.2.6), seeds were surface sterilized followed by plating on selection medium. Approximately 40 mg of seeds were placed in an Eppendorf tube containing 200 µL of 70% ethanol and constantly shaken for 2 min. The seeds were washed thoroughly by adding sterile water, vortexing and draining. After repeating this step for 3-4 times, seeds were treated for 10 min. with 600 µL of 100% bleach containing 0.1% polyoxyethylene sorbitan monolaurate (Tween 20). They were washed several times using sterile water and finally resuspended in 0.1% sterile agarose before plating on Petri plates containing ½ MS medium (M-5519, Sigma) supplemented with 0.8% agarose and 25 µg ml^{-1} hygromycin. Plates were maintained for 7-10 days at short day conditions (9h light, 150 µE m^{-2} sec^{-1}) before transferring seedlings to soil.

For seed production, plants were grown in 4" pots containing All-Purpose Mix soil (Greenleaf products Inc., Winnipeg, Manitoba, Canada). These plants were initially maintained for 4-5 weeks at short-day conditions and later transferred to long-day conditions (14h light, 100-200 µE m^{-2} sec^{-1}) to set seed.

For disease testing, seeds were first surface sterilized, incubated for three days at 4°C in Petri plates containing selection medium (where appropriate) and later transferred to a short day growth cabinet. After 10 days, seedlings were moved to soil (in pots) and maintained at short-day conditions for 3-4 weeks prior to disease testing. All the soil-grown *Arabidopsis* plants were fertilized once a week with a solution of 3 g L^{-1} of 20-20-20(N:P:K).

3.2.2. *Brassica napus* mRNA isolation and cDNA synthesis

Five-day-old *B. napus* seedlings (variety Westar) were treated with 2 mM SA for 18 h and tissue was harvested, frozen in liquid nitrogen and stored at –80°C prior to

isolation of total RNA using RNEasy kit (Qiagen, Valencia, CA, USA). mRNA was enriched from total RNA using Poly A T tract mRNA isolation kit (Promega, Madison, WI, USA) and later cDNA synthesis was performed using the 3' RACE kit (Invitrogen, Carlsbad, CA, USA). All procedures were performed according to manufacturer's instructions.

3.2.3. Polymerase chain reaction

For amplification of the *B. napus DIR1*-related sequence, the P1 forward primer including the start site (5'-ATGTCGACCGAGGATAAAATGGCGAGTAAG-3') and P2 reverse primer including the stop site (5'-ATGGATCCGCTTAACAAGTTGGAGCGTTGG-3') were designed based on the expressed sequence tag (EST) ML4307 obtained from the Agriculture and Agri-Food Canada (AAFC), Saskatoon Research Station database. P1 and P2 oligonucleotide primers were designed to incorporate SalI and BamHI restriction sites, respectively. Using *B. napus* cDNA as a template, PCR amplification (35 cycles) was performed using Exo-Taq polymerase (Stratagene, La Jolla, CA, USA) including denaturation at 94°C for 1 min, annealing at 55°C for 1 min and elongation at 72°C for 2 min and 30 sec.

3.2.4. Similarity searches and alignments

Similarity searches were performed with the internet-based facility, basic local alignment search tool (BLAST; http://www.ncbi.nlm.nih) and sequence alignments were performed using the Align X module of the Vector NTI program (Advance 10; Invitrogen, Carlsbad, CA, USA).

3.2.5. Generation of transgenic *Brassica napus* plants

The full-length coding regions of the *Arabidopsis DIR1* (AT5G48485) and the *B. napus DIR1*-related gene, including the signal peptides, were amplified by PCR from genomic DNA and cDNA, respectively, and separately inserted into a pUC19-based cloning vector under the control of the tobacco constitutive promoter (tCUP; Foster et al., 1999) and the *nopaline synthase* (*nos*) terminator. The chimeric genes were then ligated into the binary Ti-plasmid vector pCAMBIA 2300 (Center for the Application of Molecular Biology to International Agriculture, CAMBIA, Inc., Canberra, ACT, Australia) which carries a chimeric CaMV35S promoter - *neomycin phosphotransferase II* (*nptII*) gene for the selection of transgenic individuals. The constructs were separately

introduced into the *Agrobacterium tumefaciens* strain GV3101 (pMP90) by electroporation. *Agrobacterium*-mediated transformation was performed on cotyledon explants of 4-day old seedlings of *B. napus* according to Tsang et al. (2003). Transformed *B. napus* plants were selected on medium containing 20 mg L^{-1} kanamycin. Rooted plants (T_0 generation) were transferred to pots for seed production and analyzed for *AtDIR1* or *B. napus DIR1*-like gene expression and copy number by northern and Southern blot analyses respectively. Transgenic plants that resulted from a single transgenic event were labeled as A and B following the transgenic event number. For example in Figure 3.6B, plant numbers 5A and 5B indicate one transformation event.

3.2.6. Generation of transgenic *Arabidopsis* plants

Wild-type *Arabidopsis thaliana* (Ws) and *dir1-1* mutant plants were transformed by the floral dip method (Clough and Bent, 1998) using *Agrobacterium tumefaciens* strain GV3101 (pMP90) harboring the *BnDIR1* fusion in pCAMBIA 1300 which carries the *hygromycin phosphotransferase* (*hpt*) and *nptII* genes for plant and bacterial transformation selection, respectively. Transformants were selected on ½ MS medium (M-5519, Sigma) containing 25 µg ml^{-1} hygromycin. Disease testing was performed on T_2 generation plants expressing various levels of *BnDIR1*.

3.2.7. Southern blot analysis

Fully expanded leaves of 3-month-old greenhouse grown *B. napus* plants or rosette leaves of 4-week-old *Arabidopsis* plants were collected, frozen in liquid nitrogen and stored at -80°C until extraction of genomic DNA using plant DNA zol (Gibco BRL, Gaithersburg, MD, USA). The DNA (10 µg) was digested with HindIII, separated on a 1% Tris-Acetate-EDTA (TAE) agarose gel, denatured and neutralized according to standard protocol (Sambrook et al., 1989) before being blotted onto a Hybond N^+ nylon membrane according to manufacturer's instructions (Amersham, Piscataway, NJ, USA). Membranes were pre-hybridized for 15 min. at 60°C using Quick Hyb solution (Stratagene, La Jolla, CA, USA) followed by 2 hrs of hybridization using the same solution containing a radioactively labeled ([α-^{32}P] dCTP) probe consisting of the entire coding region of *AtDIR1* or the *B. napus DIR1*-related gene. Radio-labeling was performed using the random primers DNA labeling kit (Invitrogen, Carlsbad, CA, USA). Membranes were washed twice at room temperature in 2X SSC, 1% SDS for 15 min and

once in 0.1X SSC and 0.1% SDS at 65 ºC for 30 min and later exposed onto a X-ray film (Sterlin Diagnostics, Newark, DE, U.S.A.) at –80ºC.

3.2.8. RNA gel blot analysis

Total RNA isolation using Trizol and northern analysis was performed as described by Liu et al. (2005) except that 5 µg of RNA sample was analyzed. Hybridization probes contained the entire coding regions of *AtDIR1* or the *B. napus DIR1*-related gene described above, as well as the *BnPR1* and *BnPR2* genes as described in Chapter 2.

3.2.9. Pathogen infection and disease resistance assays

Disease testing of *B. napus* using *P. syringae* (van Hall) pv. *maculicola* (*Psm*) and *Leptosphaeria maculans* (Desm.) Ces. & de Not. [anamorph *Phoma lingam* (Tode ex Fr.) Desm.] was performed on 3-4 week old plants and 8-day old seedlings, respectively, as described in Chapter 2. For disease testing in *Arabidopsis*, 3 to 4 week-old plants were either sprayed with water or pre-inoculated with 1 x 10^6 cfu ml^{-1} of avirulent *P. syringae* pv. *tomato* (*Pst*) DC3000 (*avrRpt2*) prior to infection with 1 x 10^5 cfu ml^{-1} of virulent *Pst* DC3000. Both strains of *Pst* were a generous gift from Dr. Robin Cameron (McMaster University, Hamilton, ON). Innocula from both avirulent and virulent bacteria were grown at 30ºC in 2YT medium containing 50 µg ml^{-1} kanamycin and 100 µg ml^{-1} rifampicin for 16 h, re-suspended in 10 mM $MgCl_2$ and diluted to the required final concentration. Three days after the secondary inoculation, samples containing eight leaf discs (4 mm in diameter) were collected from individual plants, ground in 0.5 ml of 10 mM $MgCl_2$, serially diluted and spread onto *Pseudomonas* Agar-F medium (Difco, Sparks, MD, U.S.A.) containing 50 µg ml^{-1} kanamycin and 100 µg ml^{-1} rifampicin. Plates were incubated for 2-3 days at 30ºC and colony numbers on each plate were recorded. The average colony forming units (cfu) per leaf disc were calculated and log-transformed data were analyzed statistically by Analysis of Variance (ANOVA), General Linear Model, as implemented in the SAS software package (SAS Institute Inc., Cary, NC, USA).

3.3. Results

3.3.1 Isolation and functional prediction of the *Brassica napus DIR1* cDNA

The sequence of an EST from *B. napus* variety DH12075 encoding a protein

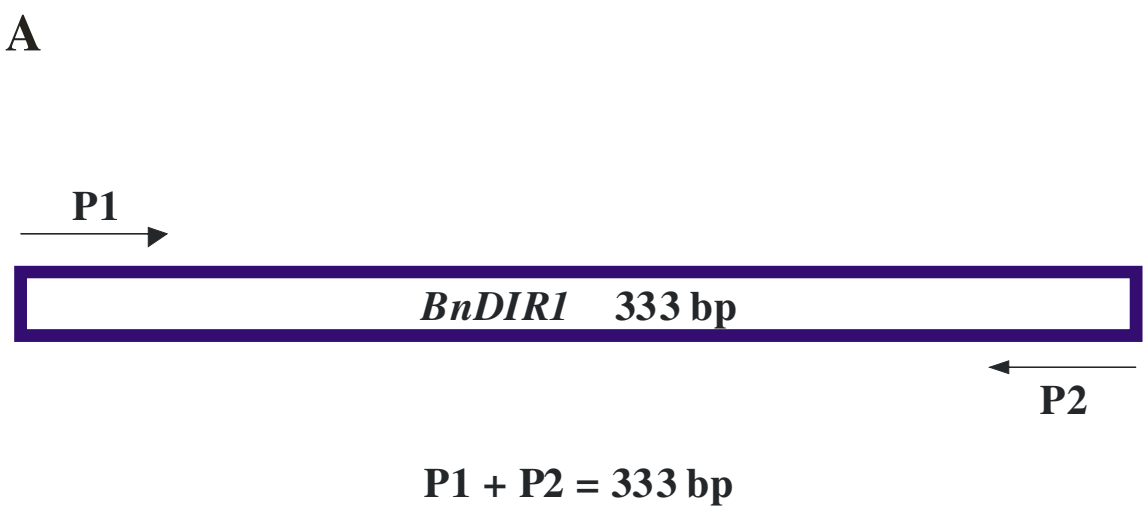
related to *Arabidopsis DIR1* was obtained from AAFC, Saskatoon Research Station. To confirm the existence of this gene (*BnDIR1*) in the *B. napus* genome and to obtain a fragment suitable for cloning purposes, the entire coding region was amplified by PCR using gene-specific primers P1 and P2 using leaf cDNA as template (Figure 3.1A). The full-length coding sequence of the product is 333 bp in length and, like *AtDIR1*, is devoid of introns. *BnDIR1* has the ability to encode a polypeptide of 110 aa with an estimated molecular weight of 11.7 kDa (Figure 3.2A). The amino acid sequence of BnDIR1 shows 71.2 % similarity to the *Arabidopsis* DIR1 and 68.3 % similarity to the predicted product of a related gene (At5G48490), closely linked to *DIR1* (Figure 3.2B). BnDIR1 contains eight cysteines that are conserved in all LTPs including AtDIR1 and the AtDIR1-like protein (Figure 3.2B). Southern blot analysis of *B. napus* genomic DNA under high stringency conditions indicated that there are at least two highly-related copies of this gene present in this species (Figure 3.6B).

3.3.2. Expression pattern of *BnDIR1* in response to pathogen challenge and BTH treatment

To determine the expression pattern of *BnDIR1,* northern blot hybridization was performed using leaves from *B. napus* four days following challenge with avirulent *Psm* 1120B (10^6 cfu ml^{-1}) or treatment with BTH (37.5 μg a.i. ml^{-1}). *BnDIR1* transcript was not detected in untreated tissue or biologically and chemically treated tissues (Figure 3.3). This indicates that *BnDIR1* is expressed in very low amounts that could not be detected by the northern blotting under the experimental conditions I used.

3.3.3. *BnDIR1* does not complement the *Arabidopsis dir1-1* mutation

It was hypothesized that if *BnDIR1* is the *B. napus* ortholog of *AtDIR1*, it should be capable of complementing the *Arabidopsis dir1-1* mutation. To this end, the coding region of *BnDIR1* was fused to the tCUP promoter and introduced into the *Arabidopsis dir1-1* background using *Agrobacterium*-mediated transformation (Figure 3.1B). Hygromycin-resistant plants were analyzed for transgene expression using the *BnDIR1* gene as a probe and individuals with a range of expression levels were identified (Figure 3.4A). The presence and copy number of T-DNA insertions in these lines were also analyzed by Southern blot analysis (Figure 3.4B). A previous study (Cao et al., 1998) demonstrated that levels of transgenic *NPR1* expression correlated with disease



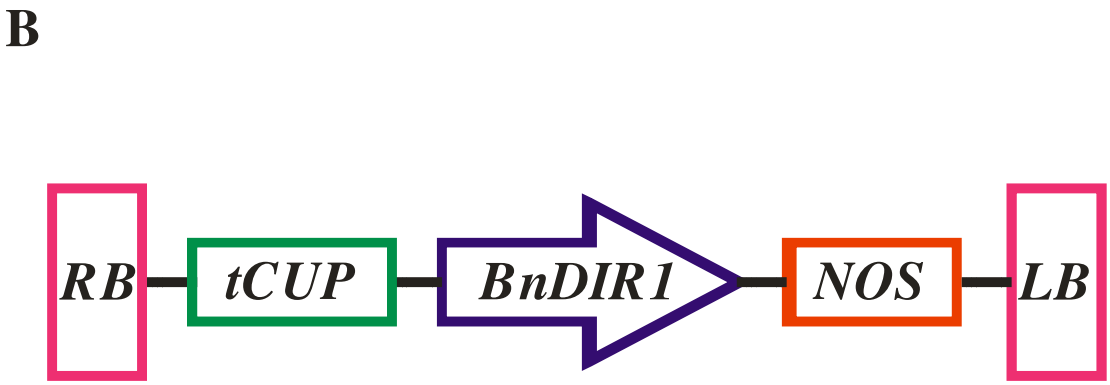


Figure 3.1A. Schematic representation of *BnDIR1* showing the primer positions used in PCR. The primer combination of P1 and P2 resulted in the expected band size of 333 bp. **3.1B.** Diagram showing the T-DNA portion of the expression vector (pCAMBIA 1300/2300 derivative) used for *Arabidopsis* and *B. napus* transformation. *RB* and *LB*, right and left T-DNA border repeats; *tCUP*, tobacco constitutive promoter with Alfalfa Mosaic Virus translational enhancer; *BnDIR1*, *B. napus DIR1 gene*; *NOS*, nopaline synthase terminator.

A

B

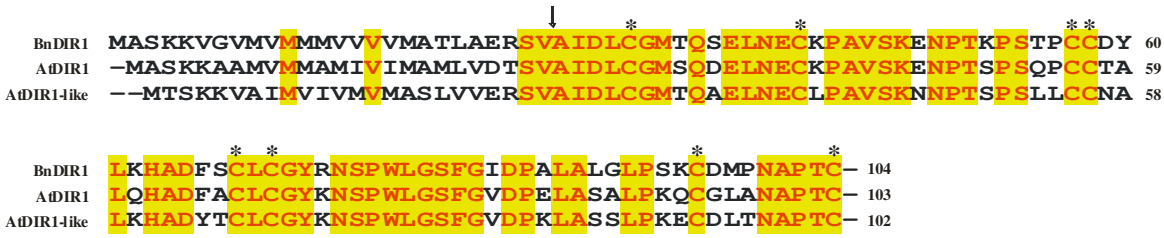


Figure 3.2A. Nucleotide (in blue) and deduced amino acid sequences (in black) of the *Brassica napus DIR1* gene. The deduced protein sequence is shown above the corresponding DNA sequence and the arrow indicates the putative signal sequence cleavage site. **3.2B.** Comparison of the deduced amino acid sequences of the predicted *Brassica napus* DIR1 (BnDIR1) and *Arabidopsis* DIR1 (AtDIR1) and *Arabidopsis* DIR1-like (AtDIR1-like) proteins. All the 8 cysteines (*) that are conserved in lipid transfer proteins are also conserved in BnDIR1. Alignment was generated using Multiple Alignment Algorithm of the Vector NTI program and begins at amino acid position 5 of BnDIR1. Identical amino acids are represented in red with a yellow background. The arrow indicates the putative signal sequence cleavage site and dashes indicate gaps introduced to maximize alignment.

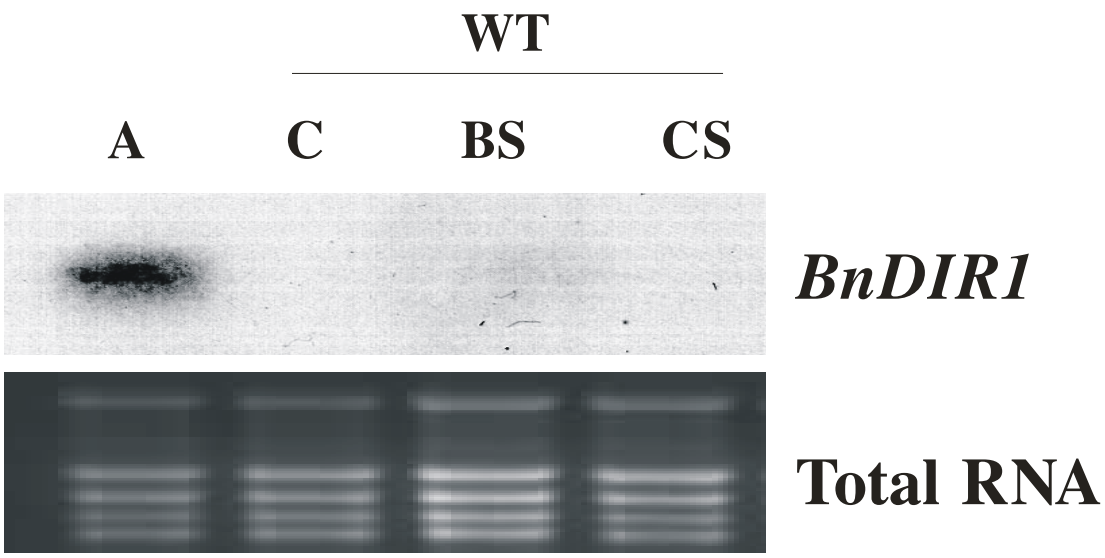


Figure 3.3. Expression of *BnDIR1* in untransformed *Brassica na*pus plants after SAR induction. First and second leaves of three-week old *B. napus* plants were infiltrated with 10 mM $MgCl_2$ (control; C) or pre-inoculated with 1 X 10^6 cfu ml^{-1} of avirulent *P. syringae* pv. *maculicola* 1120B (biological SAR; BS). Alternatively, plants were sprayed with 37.5 µg a.i. ml^{-1} BTH (chemical SAR; CS). Four days later the 3rd and 4th leaves of each plant were collected for RNA. Transgenic *Arabidopsis dir1-1* plants expressing *BnDIR1* under the control of the tCUP promoter (A) were used as a positive control. Five µg of total RNA was extracted from leaves, blotted on a nylon membrane and hybridized with radioactively labeled, full-length *BnDIR1*. A photo of the ethidium bromide-stained gel is included as a measure of RNA loading.

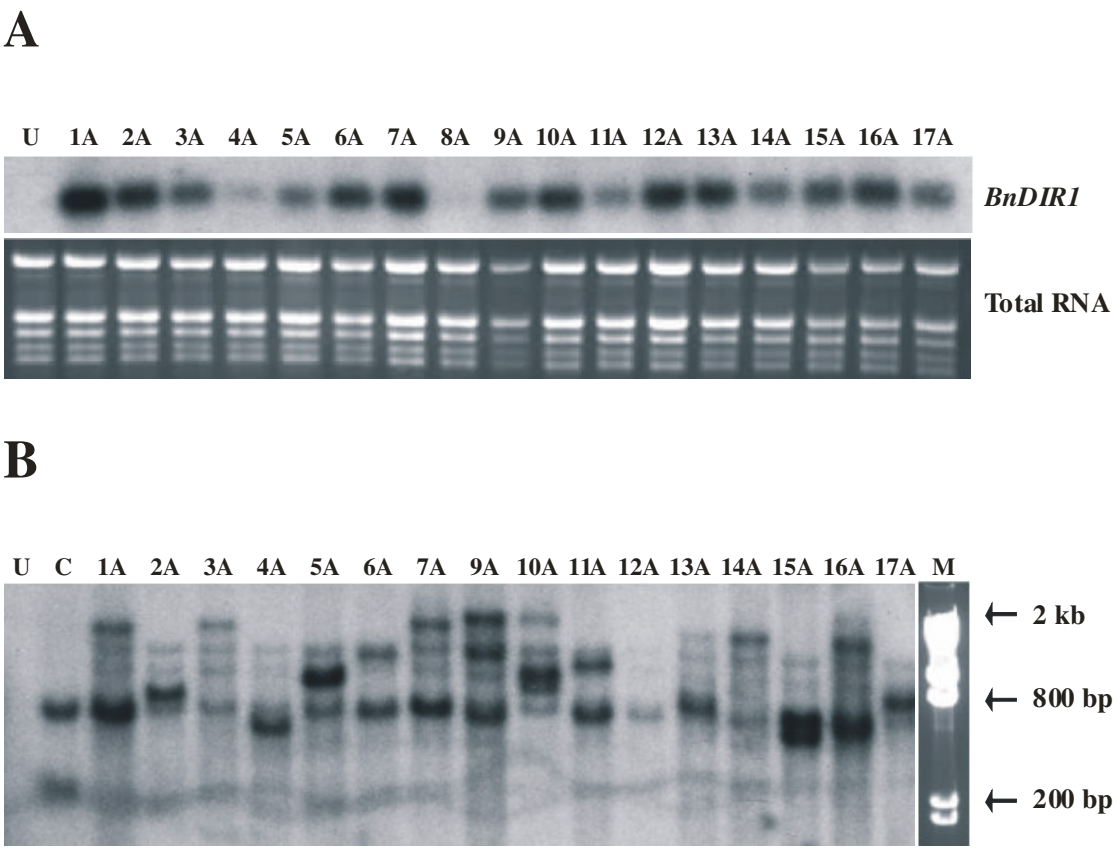


Figure 3.4A. Expression of *Brassica napus DIR1* transgene (T_2 generation) in *Arabidopsis dir1-1* mutant plants. Five µg of total RNA was extracted from leaves, blotted on a nylon membrane and hybridized with radioactively labeled, full-length *BnDIR1* coding region. A photo of the ethidium bromide-stained gel is included as a measure of RNA loading. U: untransformed *Arabidopsis* plant; 1A through 17A: *dir1-1* mutant plants expressing *BnDIR1* transgene. **3.4B.** Southern blot analysis of transgenic *Arabidopsis dir1-1* plants expressing the *BnDIR1* transgene. Genomic DNA (10 µg) isolated from untransformed *Arabidopsis*, *Brassica napus* and 16 independent transgenic lines expressing the *BnDIR1* gene was digested with HindIII and hybridized with radioactively labeled the full-length *BnDIR1* coding region as probe. U: untransformed Wassilewskija ecotype of *Arabidopsis*; C: untransformed *Brassica napus*; 1A through 17A: independent transgenic lines in *Arabidopsis dir1-1* mutant background.

C

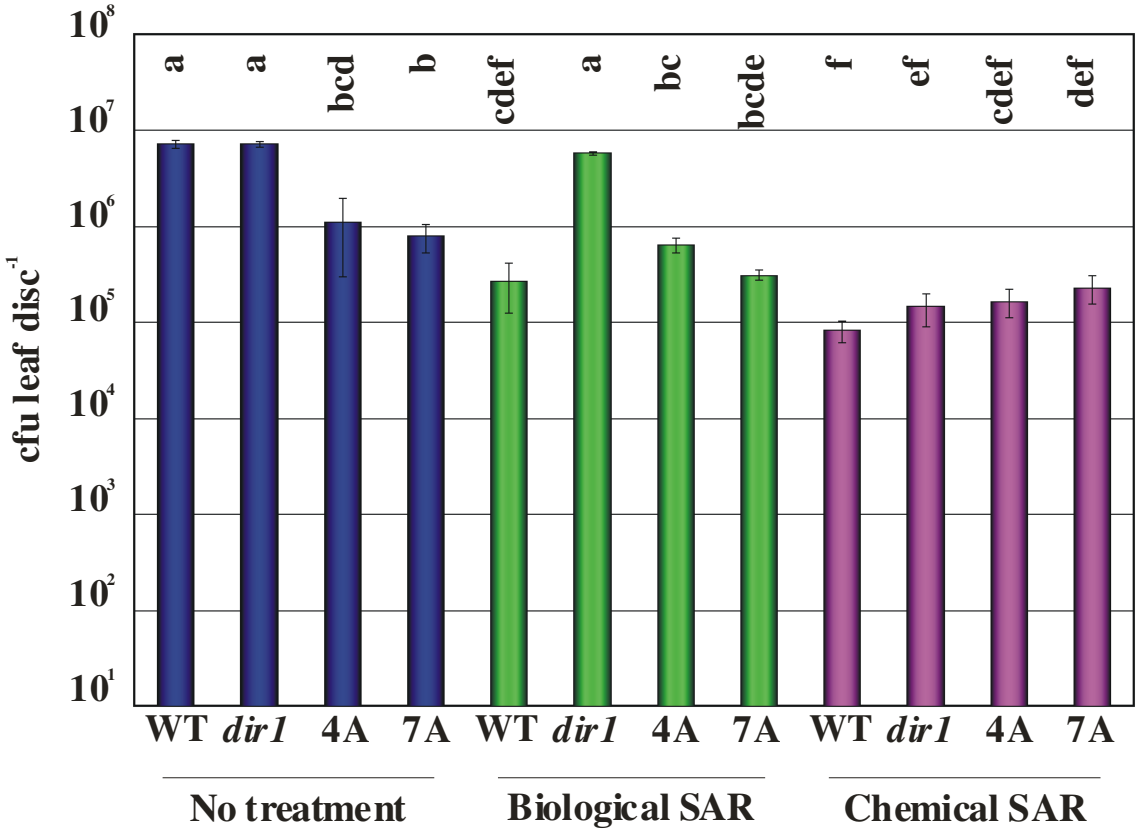


Figure 3.4C. Growth of *P. syringae* pv. *tomato* DC3000 in *Arabidopsis* wild-type, *dir1-1*, and *dir1-1* mutant plants (T_2 generation) expressing *Brassica napus DIR1* transgene. All plants are in the *Arabidopsis* Wassilewskija ecotype. Plants were either pre-inoculated with 1 X 10^6 cfu ml^{-1} of avirulent *P. syringae* DC3000 (Biological SAR) or treated with 37.5 µg ml^{-1} BTH (Chemical SAR) prior to infection with virulent *P. syringae* DC3000 (1 X 10^5 cfu ml^{-1}). Bacterial counts (cfu leaf $disc^{-1}$) were determined three days after infection. Each sample consisted of 8 leaf discs from one single plant and every data point represents the mean ± SE of 3 samples. Letters above the bars indicate treatments that are statistically the same by ANOVA analysis of log-transformed means.

resistance. Furthermore, transgene expression levels have been both positively and negatively correlated with T-DNA copy number (Hobbs et al., 1993). Accordingly, the two lines chosen for further study differed for both these parameters. Line 4A contains a single T-DNA insertion and expresses low levels of the transgene, while line 7A contains multiple inserts and expresses high levels of the transgene.

The *dir1-1* mutation was created by mutagenizing *Arabidopsis* plants using T-DNA as a mutagen (Maldonado et al., 2002). Mutant plants are specifically compromised in the SAR pathway; following pre-inoculation with an avirulent pathogen, they are more susceptible to subsequent infection by virulent pathogens when compared to wild-type plants and do not express *PR* genes in the systemic leaves (Maldonado et al., 2002). The phenotype of the *dir1-1* plants is described in more detail in Chapter 1 (section 1.7.2).

I first analyzed whether expression of *BnDIR1* affected basal resistance of the *dir1-1* mutant. Wild-type *Arabidopsis* (ecotype Ws), *dir1-1*, and T_2 generation plants from transgenic lines 4A and 7A were inoculated with 1 X 10^5 cfu ml^{-1} of virulent *Pst* DC3000. Disease resistance was assessed by quantifying viable bacteria in infected leaves (Figure 3.4C). The wild-type plants and *dir1-1* mutants displayed similar levels of bacterial growth, which were not statistically different as determined by *t*-test within analysis of variance (ANOVA). This result confirms that the *dir1-1* mutation does not affect basal resistance, as previously reported (Maldonado et al., 2002). The transgenic *dir1-1* plants expressing the *BnDIR1* gene displayed a 6.4- to 9.1-fold reduction in bacterial growth compared to the non-transformed mutant plants (Figure 3.4C). These differences were found to be statistically significant, indicating that expression of *BnDIR1* enhances basal resistance in *Arabidopsis dir1-1* plants.

I next tested whether expression of *BnDIR1* could complement the SAR defect in *dir1-1* plants. Three days prior to inoculation with virulent *Pst* DC3000, wild-type, *dir1-1* and transgenic lines 4A and 7A were inoculated with an avirulent strain of *Pst* DC3000. This treatment resulted in 27-fold reduction in bacterial growth in leaves of wild-type plants, but only a modest 1.25-fold reduction in bacterial growth in leaves of *dir1-1* plants (Figure 3.4C). The difference in bacterial growth in the wild-type was significantly different than the non-treated control, indicating deployment of SAR. However, the difference in bacterial titres between *dir1-1* plants pre-inoculated with avirulent *Pst*

DC3000 and those receiving no pre-treatment was not statistically significant, confirming the published report that *dir1-1* is defective in SAR (Maldonado et al., 2002). Titres of virulent bacteria in transgenic *dir1-1* plants expressing *BnDIR1* and pre-inoculated with the avirulent pathogen were significantly lower than those observed in the *dir1-1* plants receiving the same pre-treatment. However, bacterial titres in the transgenic lines receiving the pre-treatment were not statistically significantly lower when compared to plants from the same transgenic lines that received no pre-treatment. Therefore, although the transgenic lines are more resistant to virulent *Pst* than the parental *dir1-1* mutant following pre-inoculation with the avirulent bacteria, growth reduction appears to be attributed to enhanced basal resistance rather than complementation of the SAR defect. Of note also is that bacterial tires in the two transgenic lines were not statistically different, regardless of pre-treatment.

Similar to wild-type plants, the *dir1-1* mutant plants are capable of mounting SAR following treatment with SA (Maldonado et al., 2002). To test the effect of expressing *BnDIR1* in conjunction with SA treatment, wild-type, *dir1-1* and transgenic plants from lines 4A and 7A were sprayed with BTH (37.5 μg a.i. ml^{-1}) three days prior to inoculation with virulent *Pst* DC3000. The wild-type and *dir1-1* mutant plants displayed 87.7 and 49.8-fold reduction in bacterial growth when compared to plants of the same genotypes that were not treated with BTH (Figure 3.4C). These differences were found to be statistically significant. The transgenic *dir1-1* plants expressing *BnDIR1* displayed 31.7 to 43.6-fold reduction in bacterial growth when compared to *dir1-1* plants that were treated with BTH. These reductions of bacterial growth were found to be statistically non-significant for both the transgenic lines. Also, there were no significant reductions of bacterial growth observed between the different genotypes treated with BTH.

3.3.4. Expression of *BnDIR1* enhances basal resistance against *Pseudomonas syringae* in wild-type *Arabidopsis*

The observation that expression of *BnDIR1* enhanced basal resistance against virulent *Pst* in the *dir1-1* mutant background prompted me to test the effects of expressing this gene in wild-type (*DIR1*+) *Arabidopsis*. Using the same binary vector depicted in Figure 3.1B, transgenic lines of *Arabidopsis* wild-type (Ws) were generated using *Agrobacterium*-mediated transformation. Northern blot hybridization confirmed

that the resulting transgenic plants expressed different steady-state levels of *BnDIR1* (Figure 3.5A). Two lines, 5A (high expressor) and 7A (low expressor) were selected for further study. In the absence of SAR-inducing pre-treatments, line 5A displayed a 58.5-fold reduction and line 7A displayed a 18.4-fold reduction in bacterial growth compared to untransformed wild-type plants (Figure 3.5B). These differences were statistically significant, indicating that expression of *BnDIR1* in wild-type *Arabidopsis* confers resistance against virulent *P. syringae*.

The ability of these transgenic plants to respond to biological SAR induction was tested by inoculating plants with avirulent *Pst* DC3000 three days prior to inoculation with virulent *Pst* DC3000. Biological induction of SAR resulted in 43.8 to 58.5-fold reduction in bacterial growth in wild-type transgenic plants expressing the *BnDIR1* gene when compared to untransformed wild-type plants that did not receive the treatment (Figure 3.5B). These differences were statistically significant. However, there was only a 1.6 to 2.1-fold reduction in bacterial growth in the transgenic plants when compared to wild-type plants that received the same biological SAR pre-treatment. These values were not statistically significant, indicating that transgenic wild-type *Arabidopsis* plants expressing *BnDIR1* did not display more biological SAR when compared to wild-type plants that received biological SAR pre-treatment.

Pre-treatment of wild-type plants with BTH resulted in statistically significant (87.7-fold) reduction in bacterial growth (Figure 3.5B). However, pre-treatment of transgenic wild-type plants expressing *BnDIR1* with BTH did not result in additional decrease in bacterial growth when compared to wild-type plants that received the same treatment. Although there is a 1.5 and 3.8-fold reduction in bacterial growth in lines 5A and 7A respectively, after BTH treatment, these values were not significantly different indicating that expression of *BnDIR1* does not increase responsiveness of wild-type *Arabidopsis* plants to BTH treatment.

3.3.5. Over expression of *BnDIR1* in *Brassica napus* results in enhanced resistance against *P. syringae* pv. *maculicola*

As reviewed in Chapter 1 (section 1.7.1) over expression of genes involved in SAR can result in enhanced resistance to disease. To determine if over expression of *BnDIR1* can confer enhanced disease resistance in *B. napus*, transgenic lines containing

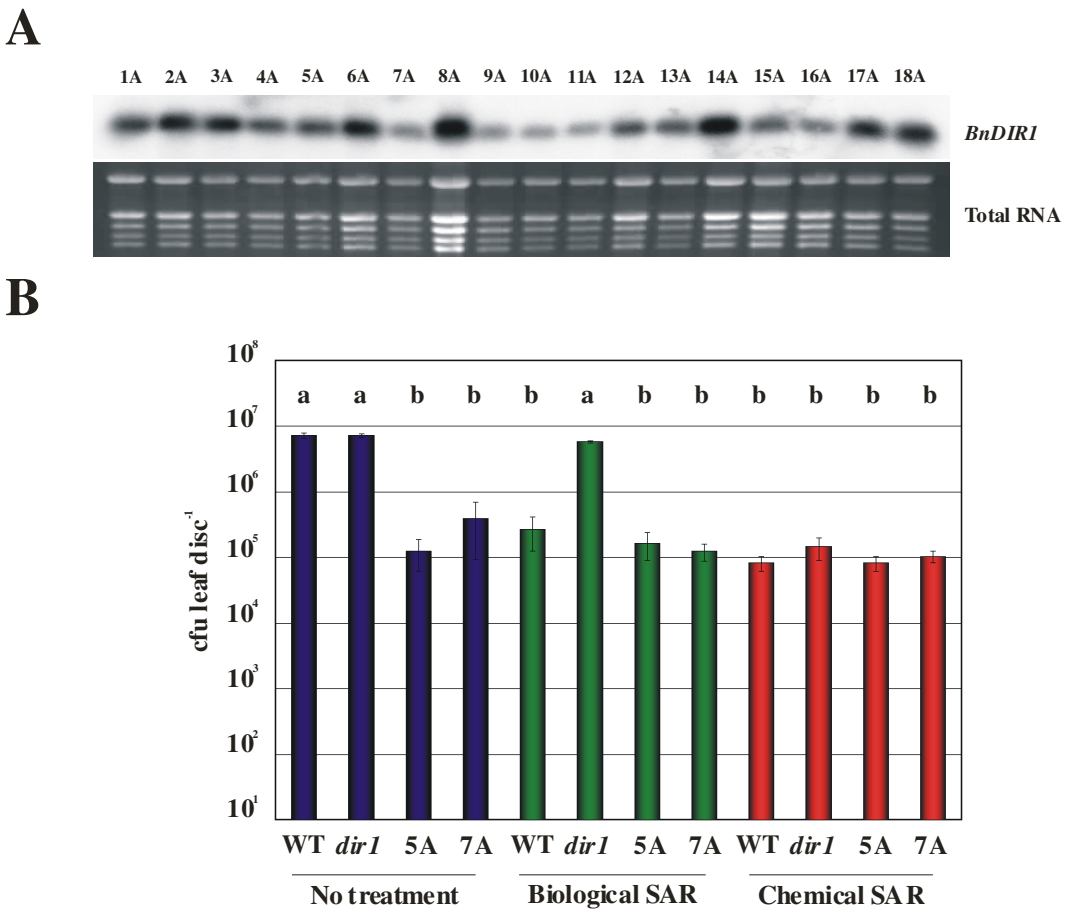


Figure 3.5A. Expression of *Brassica napus DIR1* transgene in wild-type *Arabidopsis* plants (T_2 generation). Five µg of total RNA was extracted from leaves, blotted on a nylon membrane and hybridized with radioactively labeled, full-length *BnDIR1*. A photo of an ethidium bromide-stained gel is included as a measure of RNA loading. Plants 1A through 17A: wild-type *Arabidopsis* plants expressing *BnDIR1* transgene. **3.5B.** Growth of *P. syringae* pv. *tomato* DC3000 in wild-type *Arabidopsis* (Wassilewskija ecotype), *dir1-1*, and wild-type plants (T_2 generation) expressing *Brassica napus DIR1* gene. Plants were either pre-inoculated with 1 X 10^6 cfu ml^{-1} of avirulent *P. syringae* DC3000 (Biological SAR) or treated with 37.5 µg ml^{-1} BTH (Chemical SAR) prior to infection with virulent *P. syringae* DC3000 (1 X 10^5 cfu ml^{-1}). Bacterial counts (cfu leaf $disc^{-1}$) were determined three days after infection. Each sample consisted of 8 leaf discs from one single plant and every data point represents the mean ± SE of 3 samples. Letters above the bars indicate treatments that are statistically the same by ANOVA analysis of log-transformed means.

the binary T-DNA vector depicted in Figure 3.1B were generated using *Agrobacterium*-mediated transformation. Northern blot hybridization confirmed that the plants recovered expressed different steady-state levels of *BnDIR1* (Figure 3.6A). Integration and copy number of the T-DNA were estimated by Southern blot hybridization (Figure 3.6B). There were no obvious developmental aberrations observed in any of these plants. Four different independent transgenic lines overexpressing *BnDIR1* (1, 3, 5A and 6) along with non-transformed controls were analyzed in this study.

Third and fourth leaves of three-week old plants were inoculated with 10^5 cfu ml^{-1} of virulent *Pst* (strain 1848B) and disease resistance was assessed by quantifying viable bacteria three days post-infection. Compared to untransformed controls, transgenic lines overexpressing *BnDIR1* displayed a 2.3 to 11-fold reduction in bacterial growth (Figure 3.6C). These differences were found to be statistically significant ($p \leq 0.05$), indicating that overexpression of *BnDIR1* in *B. napus* is capable of enhancing disease resistance against virulent *P. syringae*. However, there was no correlation between transgene expression and levels of resistance achieved.

As shown in Chapter 2, SAR induced by pretreatment with an avirulent pathogen or the SA analog BTH can enhance resistance of *B. napus* plants to virulent *Psm* and *L. maculans*. To test whether these SAR pre-treatments could further enhance resistance to *Psm* observed in the transgenic lines expressing *BnDIR1*, plants were either pre-inoculated with 10^6 cfu ml^{-1} avirulent *Psm* 1120B (Biological SAR) or sprayed with 37.5 a.i. mgL^{-1} BTH (Chemical SAR) prior to infiltration 4 days later with virulent *Pst* 1848B. Biological induction of SAR in the transgenic material resulted in 11.6 to 18.5-fold reduction in bacterial growth when compared to untransformed plants that did not receive a pre-treatment (Figure 3.6C). There was only a 1.2 to 2-fold reduction of bacterial growth when compared to wild-type plants that received the biological SAR induction. Nevertheless, except for line # 1, all the remaining transgenic lines showed significant differences compared to wild-type plants that received the same treatment. Chemical induction of SAR in the transgenic lines resulted in 458 to 1550-fold reduction in bacterial growth when compared to wild-type untransformed plants that did not receive any treatment (Figure 3.6C). All these reductions in bacterial growth were statistically significant. There was a 2.2 to 7.5-fold reduction in bacterial growth when compared to

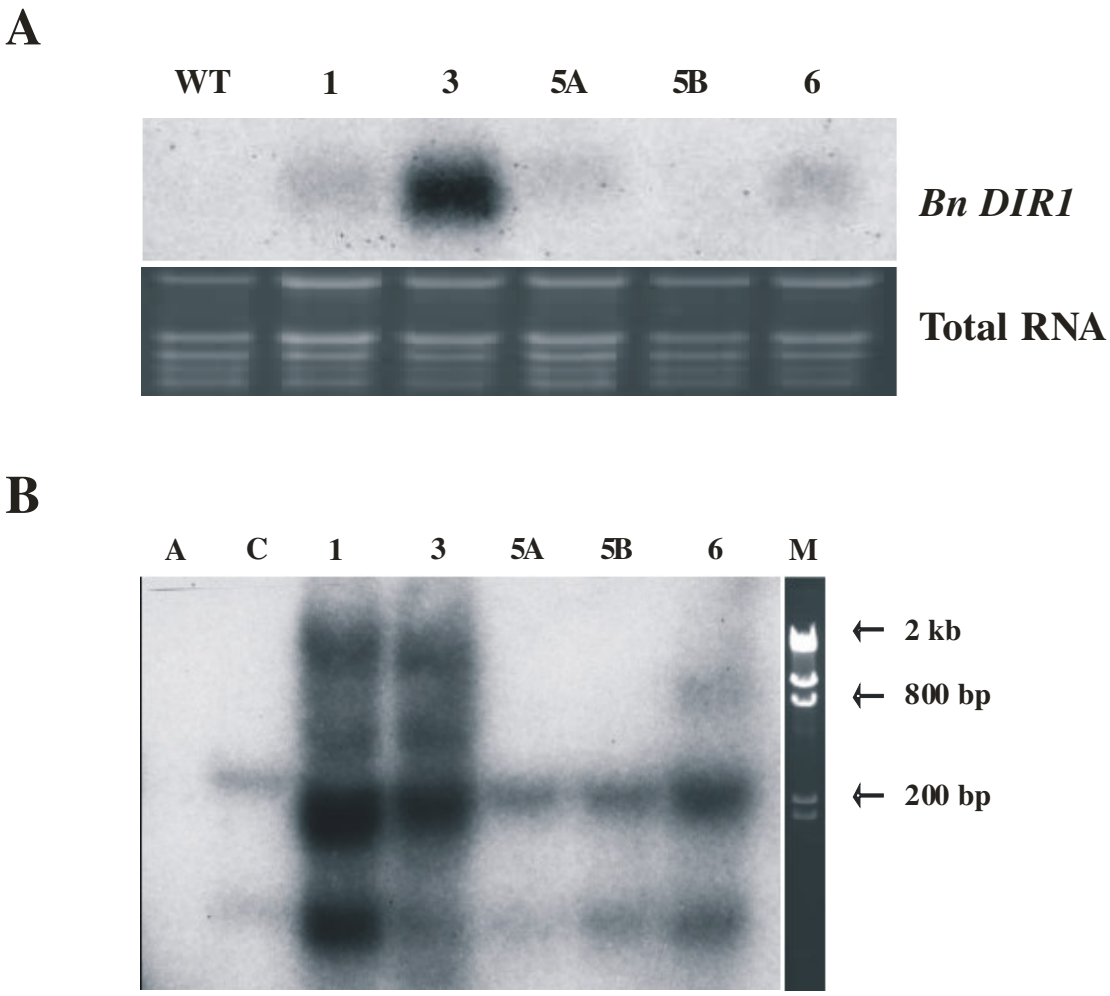


Figure 3.6A. Northern blot analysis of T_0 transgenic *Brassica napus* plants expressing *Brassica napus DIR1* coding region under the control of the tobacco constitutive promoter (tCUP). Total RNA was isolated from 4-week old plants and five µg was separated on a gel before transferring to a nylon membrane. Hybridization was performed using radioactively labeled full-length *BnDIR1* as indicated. Photographs of ethidium bromide-stained gels are included as a measure of RNA loading**. 3.6B.** Southern blot analysis of transgenic *Brassica napus* plants expressing *B. napus DIR1* coding region. Genomic DNA (10 µg) isolated from wild-type and four independent transgenic lines expressing *BnDIR1* gene was digested with HindIII and hybridized with radioactively labeled full length *BnDIR1* probe. A: *Arabidopsis* wild-type; C: untransformed *B. napus*; 1, 3, 5A, 5B and 6: transgenic lines in *B. napus* Westar background; M: molecular marker. Plants 5A and 5B are a result of a single transformation event.

C

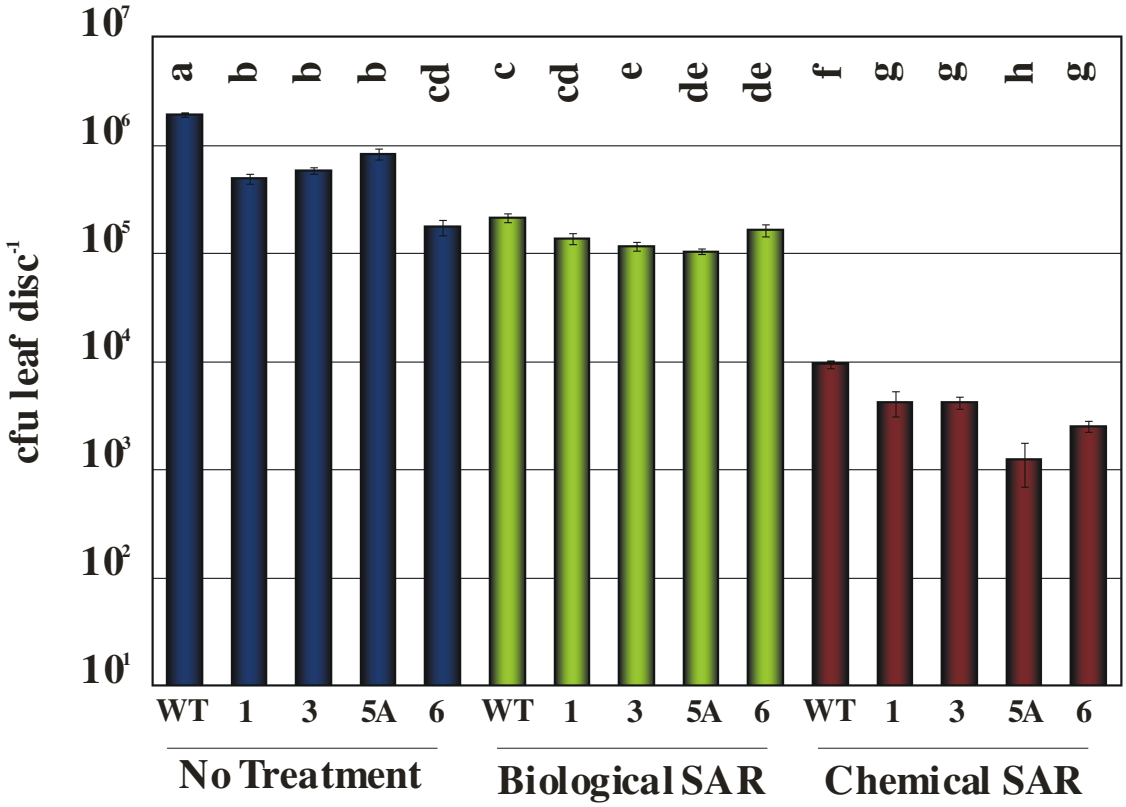


Figure 3.6C. Growth of *P. syringae* pv. *maculicola* 1848B in transgenic *Brassica napus* plants (1, 3, 5A and 6) over expressing *Brassica napus DIR1* coding region. There week old T_1 transgenic plants were either pre-inoculated with 1 X10^6 cfu ml^{-1} of avirulent *P. syringae* pv. *maculicola* 1120B (Biological SAR) or treated with 37.5 µg ml^{-1} BTH (Chemical SAR) prior to infection with virulent *P. syringae* 1848B (1 X 10^5 cfu ml^{-1}). The level of bacterial growth (cfu leaf $disc^{-1}$) was quantified four days after inoculation using serial dilution. The data points represent the mean +/- SE from 6 individual wild-type (WT) and transgenic plants. Letters above the bars indicate treatments that are statistically the same by ANOVA analysis of log-transformed means. This experiment was repeated once with similar results.

wild-type plants that received chemical SAR treatment. All of these values are statistically different. Furthermore, they are all statistically different from values obtained in the corresponding transgenic lines without SAR treatment, or after biological SAR treatment. Overall, these results indicate that combining overexpression of *BnDIR1* with SAR pre-treatments (biological or chemical), in *B. napus*, result in greater disease resistance than either alone.

3.3.6. Generation and molecular characterization of transgenic *Brassica napus* plants expressing the *Arabidopsis DIR1* gene

To test whether *AtDIR1* expression leads to enhanced resistance to disease in *B. napus*, the complete coding region under the control of *tCUP* (Figure 3.7A) was introduced into *B. napus* plants using *Agrobacterium*-mediated transformation. Thirty seven independently transformed T_0 transgenic plants were generated and grown to maturity. Northern blot analysis confirmed expression of the transgene in multiple T_0 plants (Figure 3.7B, see also Figure 3.9A). Under the hybridization conditions used, the probe did not detect expression of endogenous *B. napus DIR1* homologs. The stable integration and copy number of the transgene were demonstrated by Southern blot analysis of Hind III-digested genomic DNA. Transgenic T_0 *B. napus* plants were found to contain between 1 and 8 copies of *AtDIR1* (Figure 3.7C). All transgenic *B. napus* plants were similar to untransformed controls and displayed no obvious aberrations in their growth or development.

3.3.7. *Brassica napus* plants expressing *AtDIR1* display enhanced resistance to the bacterial pathogen *Pseudomonas syringae* pv. *maculicola* but not the fungal pathogen *Leptosphaeria maculans*

To test whether expression of *AtDIR1* could confer enhanced resistance to disease in *B. napus* plants, three different independent transgenic lines (No. 15, 50 and 89) along with non-transformed controls were infected with the virulent bacterial pathogen *Psm*

1848B. In addition to expressing different steady-state levels of *AtDIR1* transcript (Figure 3.7B), these plants harbor different copy numbers of the transgene (Figure 3.7C). Third and fourth leaves of three-week old plants were inoculated with 1×10^5 cfu ml^{-1} of *Psm* 1848B and disease resistance was assessed by quantifying viable bacteria in these leaves 3 days later. When compared to untransformed controls, transgenic lines displayed

A

B

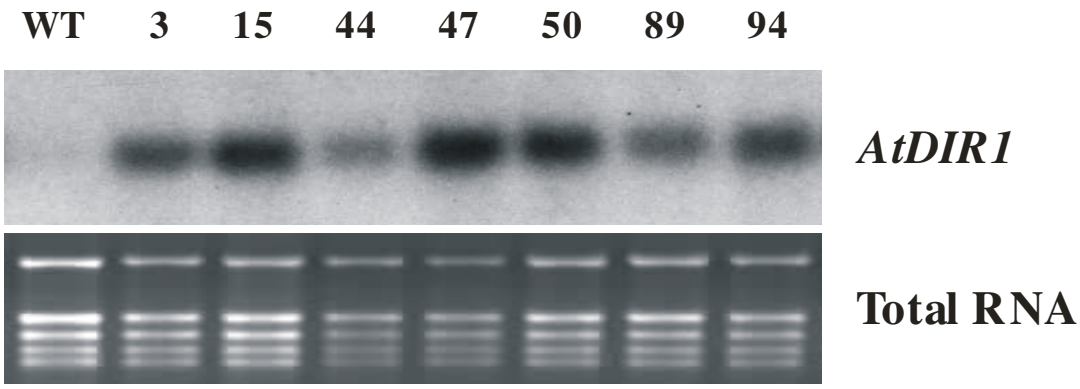


Figure 3.7A. Diagram showing the T-DNA portion of the expression vector (pCAMBIA 2300) used for *Brassica napus* transformation. *RB* and *LB*, right and left T-DNA border repeats; *tCUP*, tobacco constitutive promoter; *AtDIR1*, *Arabidopsis DIR1* gene; *NOS*, nopaline synthase terminator. **3.7B.** Northern blot analysis of T_0 transgenic *Brassica napus* plants showing various levels of *AtDIR1* transcript. Total RNA was isolated from 4-week-old plants and five µg was separated on a gel before transferring to a nylon membrane. Hybridization was performed using a radioactively labeled *AtDIR1* probe. Photos of ethidium bromide-stained gels are included as a measure of RNA loading.

C

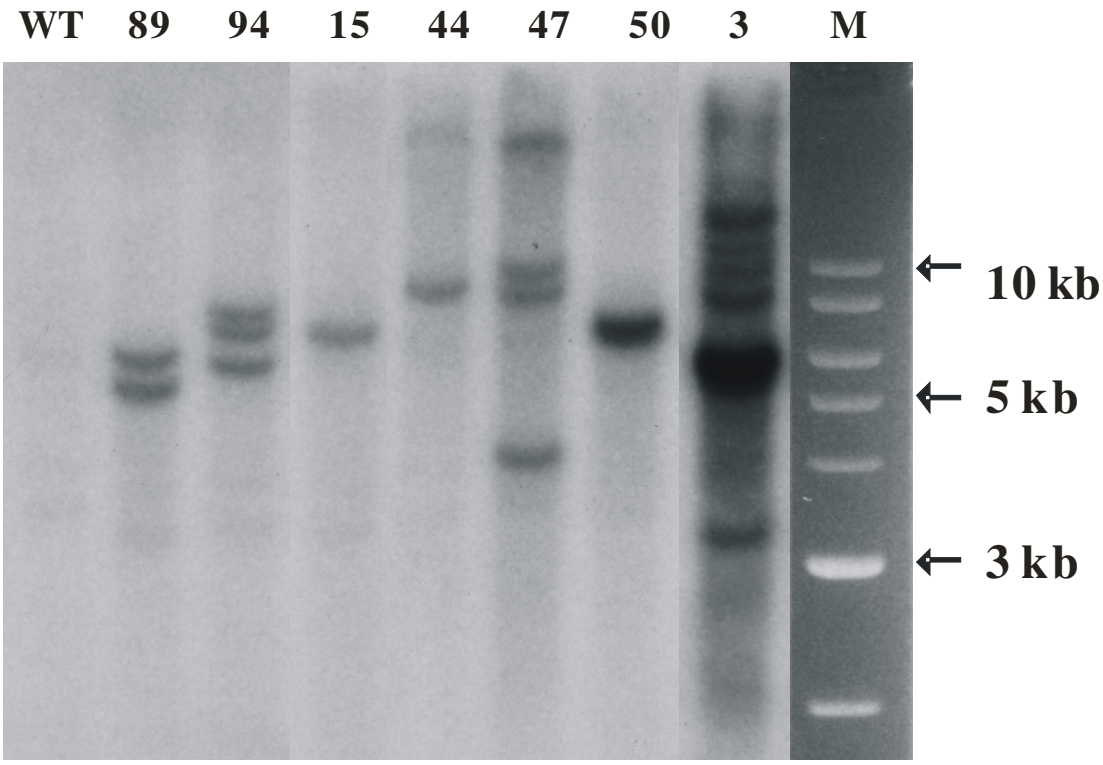


Figure 3.7C. Southern blot analysis of transgenic *Brassica napus* plants expressing *AtDIR1*. Genomic DNA (10 µg) isolated from wild-type and seven independent transgenic lines expressing *AtDIR*1 gene was digested with HindIII and hybridized with radioactively labeled full length *AtDIR1* probe. WT: wild-type; 89, 94, 15, 44, 47, 50 and 3: independent transgenic lines in *B. napus* Westar background; M, molecular marker.

a 15-22 fold reduction in bacterial growth (Figure 3.8A, blue bars). These differences were found to be statistically significant at $p \leq 0.05$ and indicate that expression of *AtDIR1* in *B. napus* is capable of enhancing disease resistance against virulent *Psm*.

Plants expressing *AtDIR1* were also tested against the fungal pathogen *L. maculans* that causes blackleg disease. Studies have indicated that *L. maculans* causes maximum damage at the seedling stage (Howlett et al., 2001). Therefore, 8-day old cotyledons from five different lines (No. 3, 15, 47, 89 and 94) were inoculated with 10 µL of *L. maculans* spore suspension ($1 \text{ X } 10^7$ ml^{-1}). The *L. maculans* strain used for disease testing (GL-11) expressed the *E. coli uidA* (*GUS*) gene under the control of the CaMV35S promoter. Therefore, qualitative and quantitative analysis of GUS activity was used to measure the fungal growth.

Quantitative analysis of GUS activity indicated that there was no reduction of fungal growth in the transgenic plants when compared to non transformed plants (Figure 3.8B, blue bars). Histological staining for GUS activity 8 days after *L. maculans* inoculation revealed that there was no difference in fungal growth in the transgenic lines when compared to untransformed control plants (data not shown). Together, these results suggest that the expression of *AtDIR1* gene in *B. napus* does not protect *B. napus* cotyledons from disease caused by the fungal pathogen *L. maculans*.

3.3.8. SAR pre-treatments in *Brassica napus* plants expressing *AtDIR1* confers greater resistance against *Pseudomonas syringae*

To test whether these SAR pre-treatments could further enhance resistance to *P. syringae* observed in the transgenic lines expressing *AtDIR1*, plants were either pre-inoculated with $1\text{X } 10^6$ cfu ml^{-1} avirulent *Psm* 1120B (biological SAR) or sprayed with 37.5 a.i. mgL^{-1} BTH (chemical SAR) prior to infiltration 4 days later with virulent *Psm* 1848B. Biological and chemical SAR treatments resulted in 10.5-364 fold reduction of bacterial growth in untransformed controls (Figure 3.8A, green and red bars, respectively). These results are statistically significant ($p \leq 0.05$) indicating effective deployment of SAR. When compared to the non-transformed control plants that received the same pre-treatments, transgenic lines expressing *AtDIR1* displayed a 2.8-4.0 fold (biological SAR) and 8.9-10.3 fold (chemical SAR) reduction in the growth of virulent bacteria. These results were statistically significant at $p \leq 0.05$.

A

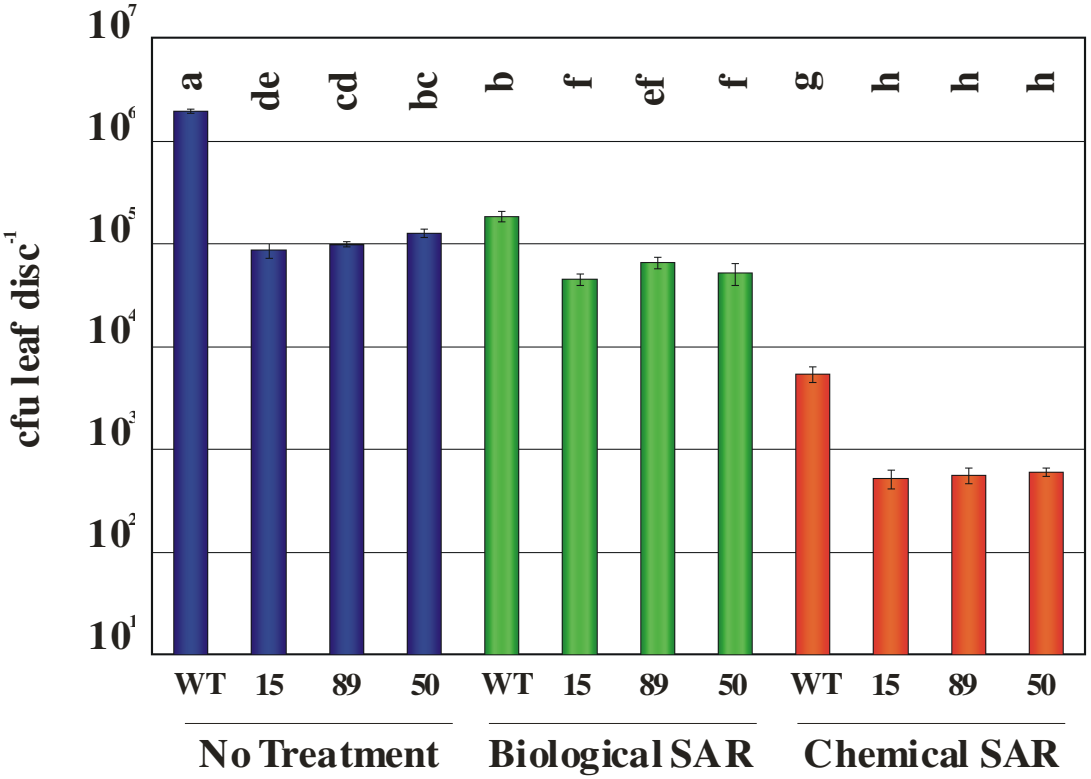


Figure 3.8A. Growth of *Pseudomonas syringae* in *Brassica napus* plants expressing *AtDIR1*. Three week old T_1 transgenic plants were inoculated with virulent *P. syringae* isolate 1848B at 1 X 10^5 cfu ml^{-1}. The level of bacterial growth was quantified four days after inoculation. Each sample consisted of 8 leaf discs and every data point represents the mean ± SE of 6 samples. Letters above the bars indicate treatments that are statistically the same by ANOVA analysis of log-transformed means. This experiment was repeated twice with similar results.

B

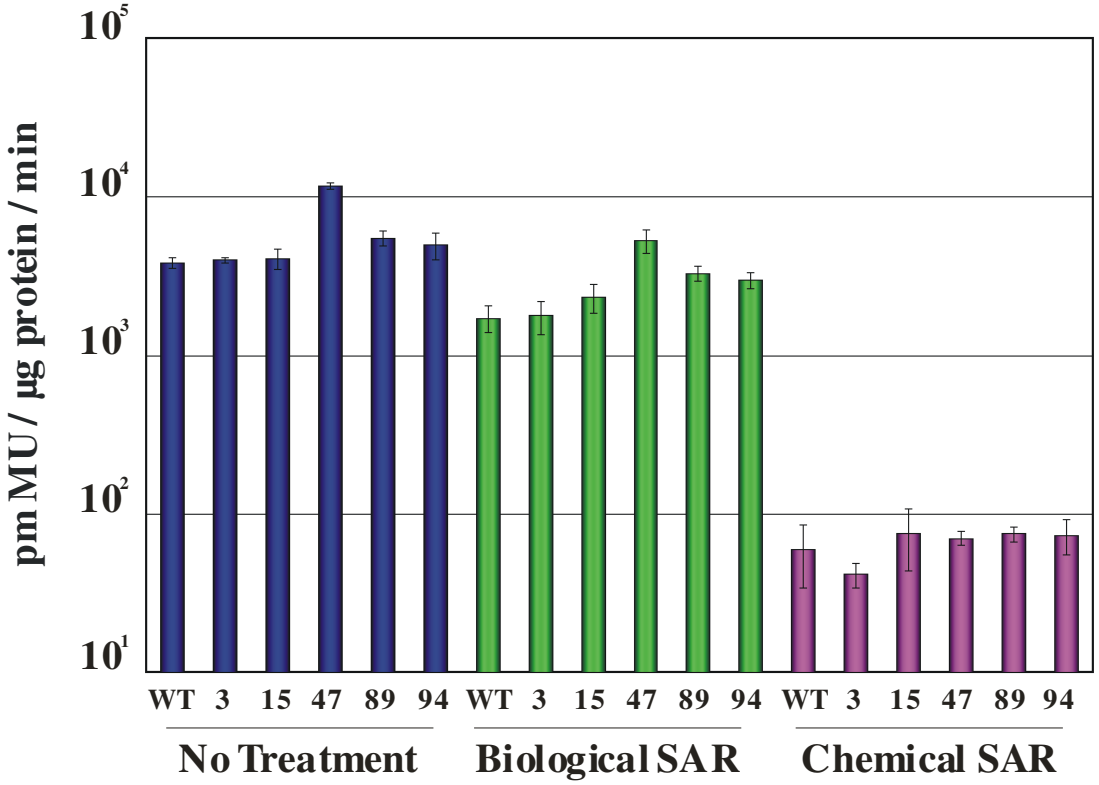


Figure 3.8B. Growth of *Leptosphaeria maculans* in *Brassica napus* seedlings expressing *AtDIR1*. Growth was assessed by quantitative analysis of GUS activity using MUG as substrate on cotyledons 8 days after inoculation with *L. maculans* and following the indicated pre-treatments. The data points in each of the figures represent the mean and standard deviation for measurements from 6 individual untransformed and transformed plants. This experiment was repeated once with similar results.

Comparisons were also made within the same transgenic line before and after SAR pre-treatments. Results indicated that there was a significant, 1.5-2.5 and 167-212 fold, reduction in bacterial growth between plants that received no pre-treatment and those that received biological and chemical SAR pre-treatments, respectively (Figure 3.8A). Thus, as observed with *B. napus* plants overexpressing *BnDIR1* (Figure 3.6C) combining *AtDIR1* overexpression with SAR pre-treatments resulted in higher levels of disease resistance.

To determine if expression of *AtDIR1* combined with SAR pre-treatments could lead to more effective resistance against *L. maculans*, eight-day old seedlings were subjected to both SAR pre-treatments. Quantitative analysis of GUS activity indicated that there was 2.2-64 fold reduction of fungal growth after biological and chemical SAR treatments, respectively, in untransformed control seedlings (Figure 3.8B, green and red bars, respectively). Similar levels of reduction (1.5-2.5 fold, biological SAR; 167-212 fold, chemical SAR) were also observed in transgenic plants expressing *AtDIR1* after SAR treatments. These results were statistically significant ($p \leq 0.05$) when compared to wild-type seedlings that did not receive any treatment, indicating the deployment of SAR. However, there was no additional reduction of GUS activity (i.e. fungal growth) in seedlings expressing *AtDIR1* when compared to non-transformed lines that received the same pre-treatments. Histological staining was also performed to monitor GUS activity in untransformed and transgenic seedlings expressing *AtDIR1* 8 days after *L. maculans* inoculation. Results from all the treatments indicated that there were no differences in fungal growth between the transgenic and untransformed seedlings (data not shown). Together, these results indicate that expression of *AtDIR1* in combination with SAR pre-treatment does not substantially enhance resistance to *L. maculans.*

3.3.9. Pathogenesis-related gene expression in *Brassica napus* plants expressing *Arabidopsis DIR1*

To elucidate the possible mechanism for enhanced resistance of *B. napus* plants expressing *Arabidopsis DIR1* against *Psm*, we monitored steady state *PR* transcript levels in several transgenic T_0 plants. Total RNA was isolated from eight-week old T_0 plants including untransformed controls and hybridized with *BnPR1*. As shown in Figure 3.9A, multiple transgenic lines expressed *BnPR1* in the absence of any pathogen challenge or

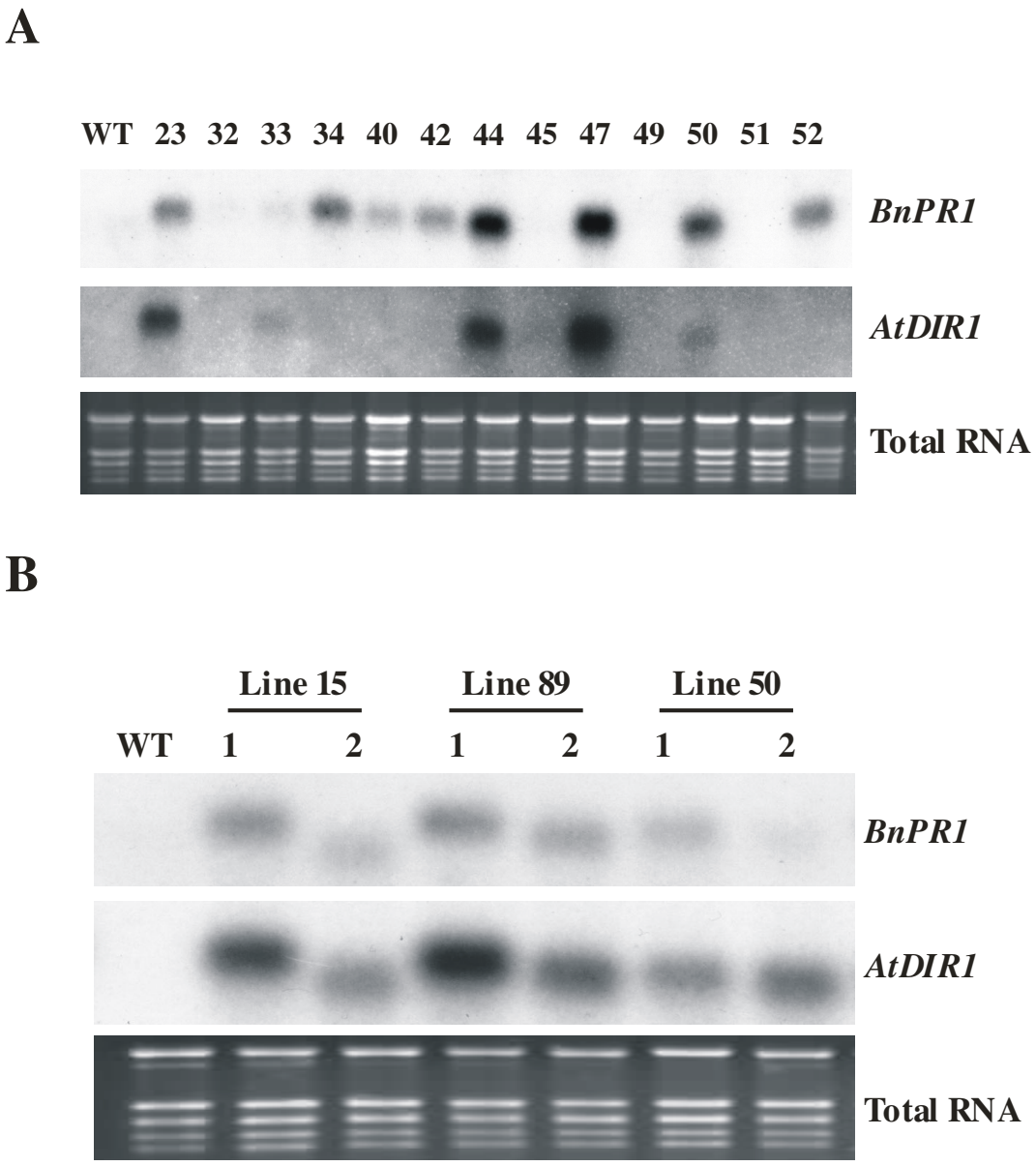


Figure 3.9. Northern blot analysis of T_0 (A) and T_1 (B) transgenic *Brassica napus* plants showing native transcript levels of *DIR1* and *BnPR1* genes. A. WT: wild-type; 23, 32, 33, 34, 40, 42, 45, 47, 49, 50, 51 and 52 independent transgenic lines in *Brassica napus* Westar background. B. WT: wild-type; Two plants each (1 and 2) from three different independent transgenic lines (15, 89 and 50). Total RNA was isolated from 4-week-old plants and five µg was separated on a gel before transferring to a nylon membrane. Hybridization was performed using a radioactively labeled *AtDIR1* probe. Photos of ethidium bromide-stained gels are included as a measure of RNA loading.

SAR pre-treatments. In general, the level of *BnPR1* correlated with those of transgenic *AtDIR1*. Lines 23, 44, 47 and 50 showed higher levels of both *AtDIR1* and *BnPR1* transcripts. However, lines such as 34, 40, 42, 50 and 52 showed very little or almost negligible levels of *AtDIR1* transcript, but exhibited intermediate levels of *BnPR1* transcript. It is noteworthy that the lack of *BnPR1* expression in some lines that express *AtDIR1* could be a detection problem and analysis with more sensitive methods (for example, quantitative real-time PCR) will be required to determine whether the transgene is expressed at low levels in these plants. Line 33 exhibited very low level of *AtDIR1* transcript although the level of *BnPR1* was almost negligible.

BnPR1 gene expression was also monitored in T_1 plants that were three to four weeks old; this developmental stage corresponds to the one used for disease testing. Similar to T_0 plants, the T_1 transgenic *B. napus* plants expressing *DIR1* exhibited varied levels of constitutive of *BnPR1* transcript (Figure 3.9B). These results demonstrate that expression of the *AtDIR1* gene in *B. napus* may be sufficient to induce the constitutive expression of *BnPR1* even without the treatment of pathogens or chemicals, and suggest that expression of *AtDIR1* may be activating defense responses. The constitutive expression of *BnPR1* in T_1 plants was observed in two different experiments; however, I could not confirm the same trend in the third replication of my experiments. Therefore, this experiment needs to be repeated before making a conclusive statement regarding the constitutive *BnPR* gene expression in *B. napus* plants expressing *AtDIR1*.

3.4. Discussion

The SAR signal transduction pathway involves three different stages. The first stage is induction or immunization where an avirulent pathogen attacks the plant leading to necrosis which in turn triggers a series of events. There is a local production of SA and the activation of *PR* genes. The last step of the induction stage is the production of a signal, the nature of which is still unknown. It has been proposed that this signal could be traveling from the site of necrosis to the systemic parts of the plants through the phloem (Jennes and Kuć, 1979).

Analysis of the *dir1-1* mutant indicates that it is defective in the production, rather than subsequent perception of the systemic signal (Maldonado et al., 2002). Pathogen challenge is needed for the induction of SAR, suggesting that the DIR1 protein needs to

be regulated or activated by an unknown process. Given that *DIR1* encodes a putative LTP, Maldonado et al. (2002) have proposed that its product may interact with a lipid-derived molecule to promote long distance signaling.

Starting from an EST sequence, I isolated the coding region for a gene encoding a DIR1-related protein from *B. napus*. The amino acid sequence of AtDIR1 and the *Brassica* protein are highly related (71% similarity), suggesting that the two proteins may be orthologous. To test this possibility, I introduced the *Brassica* coding region, under the control of a constitutive promoter, into the *Arabidopsis dir1-1* mutant background. When compared to control transgenic plants, those pre-inoculated with an avirulent pathogen did not show a substantial reduction in the growth of virulent *P. syringae*, indicating that the SAR defect was not complemented by *BnDIR1*. However, data on SAR in the transgenic plants need to be interpreted with caution, as these displayed enhanced basal resistance to virulent *P. syringae*. It is possible that this enhanced basal resistance masked the SAR response.

Arabidopsis dir1-1 mutant plants do not accumulate *PR-1* gene transcripts in systemic leaves following inoculation with an avirulent pathogen (Maldonado et al., 2002). Accordingly, I sought to determine whether there was a difference in *PR-1* transcript accumulation between the *dir1-1* mutant and the transgenic plants. However, I had difficulty in establishing conditions for obtaining differential *PR* gene expression between wild-type and *dir1-1* plants, and this line of research was abandoned. *PR* gene expression analysis in the systemic leaves of transgenic *dir1-1* plants expressing *BnDIR1*, after plants had been inoculated with an avirulent pathogen, would have provided an alternative means of assessing whether *BnDIR1* was capable of complementing the *Arabidopsis dir1-1* mutant.

The observation that expression of *BnDIR1* in the *dir1-1* mutant enhanced basal resistance was not expected, as overexpression of *AtDIR1* in this genetic background was not reported to have the same consequence (Maldonado et al., 2002). Upon closer inspection of the data of Maldonado et al. (2002), it is apparent that overexpression of *AtDIR1* is capable of enhancing basal resistance in *Arabidopsis*, as these authors report a small but significant reduction of basal resistance to *Pst* DC3000 in 1 of 6 trials. I have performed disease tests on the transgenic *dir1-1* plants expressing *BnDIR1* only once and

results will need to be confirmed in replicate trials. However, expression of *BnDIR1* in wild-type *Arabidopsis* and expression of *AtDIR1* or *BnDIR1* in *B. napus* both resulted in the enhancement of basal resistance to virulent strains of *P. syringae*. Together these findings indicate that increasing the expression of *DIR1* can enhance basal resistance to this bacterial pathogen.

It is not clear why my results differ from those of Maldonado et al. (2002). It is possible that AtDIR1 and BnDIR1 may have distinct functions, implying that they are not orthologous. Alternatively, the discrepancies may be attributed to differences in plant growth conditions, inocula preparation or infection procedures used in the two studies, or to differences in transgene expression levels and/or patterns. Of note, different promoters (CaMV35S and tCUP) were used in the two studies. An intriguing possibility is that the differences may be attributed to the fact that I tested *AtDIR1* and *BnDIR1* in heterologous hosts, which may have resulted in slight differences in the regulation of DIR1 activity.

My results indicate a positive regulatory role of *DIR1* in mediating basal disease resistance. It has been proposed that disease occurs not due to the lack of genetic information required to deploy a resistance response, but rather as a result of the inability of the plant to express the information soon enough and with sufficient magnitude to combat the pathogen (Kuć, 1982). Therefore, it is quite plausible to suggest that the mechanism for enhanced resistance against *P. syringae* in plants over expressing *AtDIR1*, without any SAR pre-treatments, may be due to the ability of these plants to perceive/produce a signal quicker and faster than the wild-type plants. This is similar to *Arabidopsis* plants over expressing *NPR1* that were resistant to virulent *Psm* without the induction of SAR (Cao et al., 1998). Consistent with this notion, my results suggest that expression of *AtDIR1* in *B. napus* plants results in constitutive *PR* gene expression. The initial analysis of *PR* gene expression was performed on material collected from eight week old T_0 plants. Although these tissues are much older than those used in the disease testing, the untransformed plants at the same age did not exhibit *BnPR1* gene expression suggesting that expression of this gene is not simply due to age-related resistance (Kus et al., 2002; Reuveni et al., 1986; Wyatt et al., 1991; Yalpani et al., 1993). Transgenic T_1 plants expressing *AtDIR1* also exhibited constitutive *BnPR1* gene expression. However, I did not see a similar trend in a third experiment and thus, results need to be interpreted

with caution and the experiment repeated. Furthermore, *PR* gene expression in transgenic *Arabidopsis* plants expressing *BnDIR1* also needs to be analyzed.

Should constitutive expression of *PR* genes in *AtDIR1* overexpressing plants be confirmed, it would suggest a mode of regulation distinct from that observed in plants overexpressing *NPR1*. For the most part, NPR1 overexpressing *Arabidopsis* plants do not constitutively express *PR* genes and instead accumulate *PR* gene transcripts more rapidly (Friedrich et al., 2001) or to higher levels (Cao et al., 1998) following pathogen challenge. A number of mutants that constitutively express *PR* genes show elevated levels of SA (Bowling et al., 1994, 1997; Clarke et al., 1998). The *dir1-1* mutant contains wild-type levels of SA (Maldonado et al., 2002), but I did not analyze endogenous SA levels in transgenic *B. napus* expressing *AtDIR1*.

The constitutive activation of defense responses including *PR* gene expression can result in detrimental phenotypes such as dwarfing and the spontaneous development of lesions. These processes could involve utilization of large amounts of energy leading to constraints in productivity of the plant (Campbell et al., 2002). None of these phenotypes were observed in *B. napus* plants expressing *AtDIR1*. Upon casual inspection, they did not exhibit any phenotypic aberrations. These plants displayed only low to moderate levels of constitutive *BnPR1* gene transcripts. It is possible that such levels can be tolerated without negative pleiotropic effects.

Several LTP-like proteins from barley and maize were shown to inhibit growth of bacterial and fungal pathogens (Molina et al., 1993), and over expression of these LTPs can enhance disease resistance in transgenic plants (Kader, 1996). Thus, I cannot rule out the possibility that at least some of the enhanced resistance observed may be due to direct antimicrobial effects of DIR1, unrelated to its signaling function.

Results presented in Chapter 2 show that SAR is effective against the black leg-causing fungal pathogen *L. maculans*. However, results presented in this Chapter indicate that the expression of *AtDIR1* in *B. napus* was not effective at enhancing resistance against this fungal pathogen. According to our earlier speculation, *AtDIR1* overexpressing plants may not be able to perceive/produce a signal quicker and faster when they are challenged with *L. maculans*. Even if this holds true, the ineffectiveness of *DIR1* over expression in providing enhanced resistance against *L. maculans* as opposed

to the effectiveness of SAR pre-treatments could be due to the fact that SAR pre-treatments may be activating several defense response pathways leading to the expression of a larger set of *PR* genes that are sufficient to combat a broad range of pathogens including *Psm* and *L. maculans*. On the other hand, *DIR1* overexpression may only be resulting in the activation of a single or fewer defense pathways that may be resulting in the activation of either a different set or a smaller number of *PR* genes resulting in fewer PR proteins that may only be sufficient to exhibit resistance against *Psm* but not *L. maculans*. Large-scale transcript profiling studies, such as microarray analysis, would be helpful in determining differential gene expression in *B. napus* plants following SAR pre-treatment as well as in *DIR1* transgenic lines.

Overall, the levels of *DIR1* transcripts detected in transgenic plants did not correlate well with the levels of *PR* transcripts in the T_0 or T_1 transgenic plants (Figure 3.9A and B). Similar observations were made by two groups that overexpressed *NPR1* in *Arabidopsis* (Friedrich et al., 2001) and tomato (Lin et al., 2004). In contrast, *Arabidopsis* plants expressing high levels of *NDR1* constitutively express *PR1* (Coppinger et al., 2004). It is possible that *DIR1* is regulated at the post-transcriptional level. Analysis of protein, rather than transcript, levels in the transgenic plants would provide a more informative basis for comparison.

To get a clear understanding of the underlying molecular mechanisms involved in plants overexpressing *DIR1* following challenge with a virulent pathogen as well as after SAR pre-treatments, it would be useful to study the *PR* gene expression in these plants before and after SAR pretreatments. I have shown that SA content increases in local and systemic leaves after SAR pre treatments in untransformed plants (Chapter 2). Therefore, analysis of transgenic *B. napus* plants expressing *DIR1* for SA content before and after SAR pre treatments may help us discern whether levels of SA play a role in enhanced disease resistance. Furthermore, it would be very interesting to test phloem exudates from my plants for their ability to induce *PR* gene expression when infiltrated into naïve leaves.

Over expression of *AtNPR1* in *Arabidopsis* was reported to enhance the responsiveness of plants to BTH resulting in increased resistance against *H. parasitica* (Friedrich et al., 2001). Results from my research indicate that transgenic *B. napus* plants

over expressing *AtDIR1* display additional disease resistance against *Psm* after chemical SAR pre-treatment suggesting the possible occurrence of priming in these plants. It will be interesting to find the lowest concentration of BTH that is required in combination with overexpression of *AtDIR1* to produce maximum effect of enhanced resistance in *B. napus* plants. Using large amount of chemicals can be very expensive for agriculture and taxing on the environment as well. Using a combination of *AtDIR1* overexpressing and BTH treatment may have benefits over the use of BTH treatment alone. It is possible that a combination of engineered plants to overexpress *AtDIR1* and BTH treatment may result in a broader range of protection against pathogens. Therefore, use of *AtDIR1* over expressing plants in combination with reduced amounts of BTH has a potential to develop new crop protection strategies against pathogens.

CHAPTER 4. Characterization of NPR1 Function in *Brassica napus*

The *Arabidopsis thaliana NPR1* (*AtNPR1*) gene is an important regulator of systemic acquired resistance (SAR). Results from Chapter 2 established that SAR in *B. napus* shares many similarities to the phenomenon reported in *Arabidopsis*. This Chapter describes the isolation and characterization of an *NPR1*-related gene from *B. napus*. Also presented is the analysis of transgenic *B. napus* plants overexpressing this gene or *AtNPR1* in response to infection by *P. syringae* and *L. maculans*.

4.1. Introduction

Systemic acquired resistance (SAR) is an induced disease resistance response that is established after the plant is exposed to a necrotizing pathogen, leading to the accumulation of SA and induction of a subgroup of pathogenesis-related (*PR*) genes also known as SAR genes (Ryals et al., 1996; Durrant and Dong 2004). SAR is known to confer broad range and long lasting resistance against several plant pathogens (Durrant and Dong, 2004; Kuć, 1982; Ryals et al., 1996; Sticher et al., 1997). SAR can also be induced by spraying SA or its functional analogs INA and BTH (Ryals et al., 1996).

Genetic analyses have identified several regulators involved in the SAR signal transduction pathway (Durrant and Dong, 2004; see also Chapter 1.7). One of these, the *Arabidopsis NON EXPRESSOR OF PR1* (*NPR1*), has been well-studied and is known to be a key positive regulator of SAR (Cao et al., 1994; Dong, 2004). Plants with loss-of-function mutations in *NPR1* cannot mount effective SAR against virulent strains of *P. syringae* or *P. parasitica* (Cao et al., 1994; Delaney et al., 1995), nor induced systemic resistance (ISR) against *P. syringae* (Pieterse et al., 1998). These mutants are also compromised in basal resistance against biotrophic pathogens such as *P.syringae*, *P. parasitica*, and *E. cichoracearum*. They have been found to be more susceptible to some incompatible races of *P. parasitica* (Delaney et al., 1995; Liu et al., 2005; McDowell et al., 2000), *P. syringae* (Shah et al., 1997), and *E. cichoracearum* (Xiao et al., 2005) but not all (Rairden and Delaney, 2002). However, loss of NPR1 function does not affect

age-related resistance against *P. syringae* (Kus et al., 2002) or basal resistance against necrotrophic pathogens, including *Alternaria brassicicola* and *Botrytis cinerea* (Thomma et al., 1998). Thus, in addition to SAR, NPR1 is required for ISR, basal resistance and signaling through some, but not all *R*-genes.

npr1 mutant plants are compromised in the expression of SA-inducible *PR* genes, including the well accepted markers of SAR, *PR-1*, *PR-2* and *PR-5*, following treatment with SA or SA analogs (Cao et al., 1994; Glazebrook et al., 1996; Liu et al., 2005). The plants accumulate high levels of SA following pathogen challenge (Delaney et al., 1995), and are hypersensitive to exogenous SA (Cao et al., 1997).

The *NPR1* gene is constitutively expressed and its levels are further elevated after treatment with SA or pathogen infection (Cao et al., 1997; Ryals et al., 1997). It encodes a novel protein containing a nuclear localization sequence (NLS) and two protein-protein interaction domains known as the ankyrin repeat domain (ARD) and the Broad Complex, Tramtrack, and Bric-a-brac/Pox virus and Zinc finger (BTB/POZ) domain (Cao et al. 1997; Sedgwick and Smerdon, 1999). Most of the *NPR1* mutations map to the ARD domain, suggesting an important function for this motif. The *npr1-2* mutation, which alters cysteine 150 to a tyrosine, maps to a non-conserved region of the BTB/POZ domain. The *npr1-3* mutant introduces a premature stop codon, resulting in a truncation of the last 194 amino acids from NPR1.

NPR1 is present both in the cytoplasm and nucleus (Després et al., 2000). Under uninduced conditions, cytoplasmic NPR1 protein exists as an oligomer, held together by intermolecular disulfides bridged between conserved cysteine residues (Mou et al., 2003). Upon SAR induction, redox changes in the cell lead to the reduction of key cysteines, resulting in the monomerization of NPR1 and its subsequent translocation to the nucleus (Mou et al., 2003). Nuclear localization of NPR1, mediated by a NLS localized near the C-terminal, is necessary for the activation of *PR* genes (Kinkema et al., 2000; Mou et al., 2003).

NPR1 is thought to activate *PR* genes by modulating the activity of a family of basic leucine zipper (bZIP) transcription factors called TGA factors (Després et al., 2000; Zhang et al., 1999; Zhou et al., 2000). These transcription factors were originally isolated as proteins capable of binding to the *activating sequence-1* (*as-1*) element of the

Cauliflower Mosaic Virus (CaMV) 35S promoter (Katagiri et al., 1989) as well as a related element in the *Agrobacterium tumefaciens* transfer-DNA (T-DNA) *octopine synthase* (*ocs*) gene (Fromm et al., 1989; Zhang et al., 1993). The *as-1* element has been shown to confer responsiveness to phytohormones such as SA, methyl jasmonate (MeJA) and auxins (Xiang et al., 1996). Of note, *as-1*-like *cis*-elements have been implicated in the regulation of the *Arabidopsis PR-1* (Lebel et al., 1998) and *GLUTATHIONE S-TRANSFERASE 6* (*GST-6*; Strompen et al., 1998) genes.TGA factors have been shown to bind specifically to the *as-1*-like elements of the *Arabidopsis PR-1* promoter in vitro (Després et al., 2000; Zhang et al., 1999; Zhou et al., 2000), while chromatin immunoprecipitation (ChIP) demonstrated that TGA2 and TGA3 bind to the *PR-1* promoter in planta, presumably on these same *as-1*-like elements (Johnson et al., 2003). The *as-1* element has also been detected in the promoters of genes that are differentially expressed during pathogen infection (Mahalingam et al., 2003).

Several studies have demonstrated physical interactions between NPR1 and members of the TGA factor family using the yeast two-hybrid system or in vitro (Després et al., 2000; Zhou et al., 2000; Zhang et al., 1999). Using an in vivo protein fragment complementation assay (PCA), Subramaniam et al. (2001) demonstrated that NPR1 and TGA2 interact in planta. The interaction between these two proteins was shown to be stimulated by SA at which point it was localized to the nucleus (Subramaniam et al., 2001). In planta interaction between NPR1 and TGA2 was also demonstrated by Fan and Dong (2002). Interestingly, a member of the TGA family (TGA1) that does not interact with NPR1 in the yeast-two hybrid assay or in vitro was shown to interact with NPR1 in *Arabidopsis* leaves following treatment with SA (Després et al., 2003). It appears as though SA-induced reduction of key cysteines in TGA1 is a prerequisite for this factor to interact with NPR1 (Després et al., 2003). Of note, NPR1 mutations that compromise disease resistance abolish interactions with TGA factors either in yeast (Després et al., 2000; Zhang et al., 1999) or in planta (Subramaniam et al., 2001) suggesting that interaction with these transcription factors is important for the biological function of NPR1.

Interaction of NPR1 enhances the binding of TGA factors to their cognate promoter elements in vitro (Després et al., 2000; Després et al., 2003). In planta, both

NPR1 and SA are required for the activation of a TGA2-dependent reporter gene (Fang and Dong, 2002). Furthermore, the binding of TGA2 and TGA3 to the *PR-1* promoter, as demonstrated by ChIP analysis, also requires NPR1 and SA (Johnson et al., 2003). Combined with the observation that simultaneous mutation of *TGA2*, *TGA5* and *TGA6* results in the loss of SA-induced *PR-1* expression and SAR (Zhang et al., 2003), these data indicate that TGA factors play an important role in the manifestation and possibly establishment phases of SAR pathway and that NPR1 is an important regulator of TGA function.

When overexpressed in the same species, the *Arabidopsis NPR1* confers enhanced basal disease resistance against *P. syringae*, *P. parasitica* (Cao et al., 1998; Friedrich et al., 2001) and *E. cichoracearum* (Friedrich et al., 2001). Most transgenic lines did not exhibit constitutive expression of *PR* genes. In one study, the increased resistance observed was correlated with a stronger, rather than faster, *PR* gene expression (Cao et al., 1998) while in another, it was associated with faster induction of *PR* genes, rather than higher levels of *PR* transcripts (Friedrich et al., 2001). In the first study, Cao et al. (1998) reported a direct correlation existed between levels of NPR1 protein and disease resistance.

Overexpression of *AtNPR1* in tomato resulted in substantial resistance against virulent strains of *P. syringae* and *Fusarium oxysporum*, moderate resistance to *Xanthomonas campestris*, *Ralstonia solanacearum* and *Stemphylium solani*, but no enhanced resistance to *Phytophthora infestans*, CMV and Tomato Yellow Leaf Curl Virus (Lin et al., 2004). This study suggested that in general, the levels of *NPR1* correlated with the effectiveness of disease resistance; however, several exceptions led the authors to suggest that resistance may require a threshold level of *NPR1* expression. They also did not find any correlation between the levels of six *PR* genes tested with either levels of *NPR1* or disease resistance.

Overexpression of *AtNPR1* in rice led to enhanced resistance against the bacterial blight-causing pathogen *Xanthomonas oryzae* pv. *oryzae* (Chern et al., 2001). These results suggest that NPR1 function and signaling is conserved in both mono- and dicotyledonous plants. It was observed that resistance to *X. oryzae* conferred by *AtNPR1* was not as effective as *R*-gene mediated resistance. However, substantial reduction of

pathogen growth was observed in the leaf central vein which limited bacterial spread and enhanced survival of the rice plants. Similar to overexpression studies in tomato, a threshold level of NPR1 was proposed to be required for resistance. Transgenic rice overexpressing *AtNPR1*, when grown under different conditions showed development of spontaneous disease-like lesions and this phenotype was correlated with the accumulation of hydrogen peroxide (Fitzgerald et al., 2004). The production of spontaneous lesions has never been reported in dicotyledonous plants overexpressing *AtNPR1* (Cao et al., 1998; Friedrich et al., 2001; Lin et al., 2004). Untransformed rice plants contain very high constitutive levels of endogenous SA whereas the transgenic plants contained lower levels of SA indicating a link between NPR1 and the regulation of SA metabolism. The transgenic *Arabidopsis* plants overexpressing *AtNPR1* responded to lower levels of BTH (SA functional analog) than the untransformed wild-type plants (Friedrich et al., 2001) thereby displaying enhanced responsiveness BTH.

Chern et al. (2005b) isolated a rice *NPR1* homolog, *NHI* and showed that when overexpressed in the same species it led to enhanced disease resistance against *Xanthomonas oryzae* pv. *oryzae*. In contrast to *NPR1* overexpressing *Arabidopsis* plants where defense genes were not activated until induced, the rice plants overexpressing *NHI* displayed constitutive expression of defense related genes. Greenhouse grown rice plants overexpressing *NHI* developed lesion-mimic spots on leaves at a pre-flowering stage. The same plants when grown in growth chambers under low light were dwarfed and contained higher levels of SA compared to wild-type plants indicating that *NHI* may be involved in the regulation of SA in response to environmental changes.

4.2. Materials and methods

4.2.1. Plant growth conditions

The different genotypes of plants that were used in this study were *Brassica napus* (L.) cv. Westar, *Arabidopsis thaliana* (L.) ecotype Columbia (wild-type), and the *Arabidopsis thaliana npr1-2* and *npr1-3* mutants (Glazebrook et al., 1996), both of which are in the Columbia genetic background. Seed for *Arabidopsis npr1-2* and *npr1-3* mutants was obtained from the *Arabidopsis* Biological Resources Center, Ohio State University**.** Conditions for surface sterilization of *Arabidopsis* seed and the growth of *Arabidopsis* and *B. napus* are detailed in Chapter 3.2.1.

4.2.2 mRNA isolation and cDNA synthesis

Five-day-old *B. napus* seedlings were treated with 2 mM SA for 18 h and tissue was harvested, frozen in liquid nitrogen and stored at -80°C prior to isolation of total RNA using RNEasy kit (Qiagen, Valencia, CA, USA). mRNA was enriched from total RNA using the Poly A T tract mRNA isolation kit (Promega, Madison, WI, USA) and cDNA synthesis was performed using the 3' RACE kit (Invitrogen, Carlsbad, CA, USA). All procedures were performed according to manufacturer's instructions.

4.2.3. Similarity searches and evolutionary analysis

Similarity searches and sequence alignments were performed with the internet-based facility, basic local alignment search tool (BLAST; http://www.ncbi.nlm.nih) and CLUSTAL W of the Megalign program of DNAStar (v6.0; Laser Gene, Madison WI, USA).

4.2.4. PCR amplification of *BnNPR1*, plasmid constructions and plant transformations

Oligonucleotide primers were designed to incorporate different restriction sites suitable for cloning. The 5' half of the *BnNPR1* gene including the start site was amplified using the P5G forward primer (5'-ATGTCGACCATGGAGACCATTGCTGGA-3') containing a SalI site and the P3P reverse primer (5'-GCATACGCTTCGTCTAGATTCGTGTGGCCCTC-3') containing a XbaI site. The 3' portion of *BnNPR1* including the stop site of *BnNPR1* was amplified using the P5J forward primer (5'-GAGGGCCACACGAATCTAGACGAAGCGTATGCTCTCC-3') containing a XbaI site and the S081430P2 reverse primer (5'-ATGGATCCTCACCGACGCCGGTGAGAGGGTTTAGC-3') containing a BamHI cloning site. PCR amplification (35 cycles of denaturing at 95°C for 1 min, annealing at 55°C for 1 min and elongation for 2 min and 30 sec at 72°C) was performed using Exo-Taq polymerase (Stratagene, La Jolla, CA, USA) and the two PCR products were inserted under the control of CaMV 35S constitutive promoter and *nopaline synthase* (NOS) terminator into the SalI and BamHI sites of the vector pFL 1480, a derivative of the binary vector pCAMBIA 2300 (Center for the Application of Molecular Biology to International Agriculture, CAMBIA, Inc., Canberra, ACT, Australia) using a three point

ligation. The identity of the plasmid construct was confirmed by sequencing using universal forward and reverse primers as well as gene specific primers. *Agrobacterium tumefaciens* strain GV3101 (pMP90) harboring the resulting plasmid was used to transform *Arabidopsis thaliana* wild-type, *npr1-2* and *npr1-3* mutant plants by the floral dip method (Clough and Bent, 1998). Transformants were selected by plating surface sterilized seeds on ½ MS medium (M-5519, Sigma) containing 50 µg ml^{-1} kanamycin. Disease testing and gene expression studies were performed on T_2 and T_3 generation plants expressing various levels of *BnNPR1*. T_2 plants are labeled by a number (representing the identity of the T_1 parent) followed by a letter (for example, 2A, 3A, 6A and so on as in Figure 4.6A) and T_3 plants are labeled by a number followed by the letter of the parental T_2 plant followed by a number (for example 2L1, 2L2, 2L3 and so on as in Figure 4.5A).

To express *Arabidopsis NPR1* (AT1G64280) and *BnNPR1* in *B. napus*, the entire coding region of *AtNPR1* and *BnNPR1* were ligated independently into pFL 1480 as described above. *Agrobacterium*-mediated transformation was performed on cotyledon explants of 4-day old seedlings of *B. napus* according to Tsang et al. (2003). Transformed *B. napus* plants were selected on medium containing 20 µg ml^{-1} of kanamycin. Rooted plants (T_o generation) were transferred to pots for seed and analyzed for *AtNPR1* and *BnNPR1* gene expression by northern analysis. Transgenic plants that resulted from a single transgenic event were labeled as A and B preceded by the transgenic event number. For example in Figure 4.7A, plant 6B indicates one transformation event.

4.2.5. Pathogen infection and disease resistance assays

Transgenic *Arabidopsis* plants containing a selection of high, medium and low expressors of *BnNPR1* were chosen for complementation studies and disease testing. For complementation studies, three- to four-week-old *Arabidopsis* plants were sprayed with 0.5 mM SA until imminent run-off and leaf tissue was collected after 18 h for northern blot analyses. For disease testing, three- to four-week-old *Arabidopsis* plants were either sprayed with water or 0.5 mM SA prior to infection with virulent *P. syringae* pv. *tomato* (*Pst*) DC3000 that was grown at 30 ºC in 2YT medium containing 50 µg ml^{-1} of kanamycin and 100 µg ml^{-1} of rifampicin for 16 h to an OD of 0.8, re-suspended in 10 mM $MgCl_2$ and diluted to a final concentration of 1 X 10^5 cfu ml^{-1}. Three days after

inoculation, samples containing eight leaf discs (4 mm in diameter) were collected from individual plants, ground in 0.5 ml of 10 mM $MgCl_2$, serially diluted and spread onto *Pseudomonas* Agar-F medium (Difco, Sparks, MD, U.S.A.) containing 50 µg ml^{-1} of kanamycin and 100 µg ml^{-1} of rifampicin. Plates were incubated for 2-3 days at 30ºC and colony numbers on each plate were recorded. The average colony forming units (cfu) per leaf disc were calculated and analyzed statistically using a matched, unpaired Students *t*-test (Witte, 1989).

Disease testing of *B. napus* using *P. syringae* pv. *maculicola* (*Psm*) and *L. maculans* was performed according to the procedures described in Chapter 2.2.4 and Chapter 2.2.5.

4.2.6. Northern blot analysis

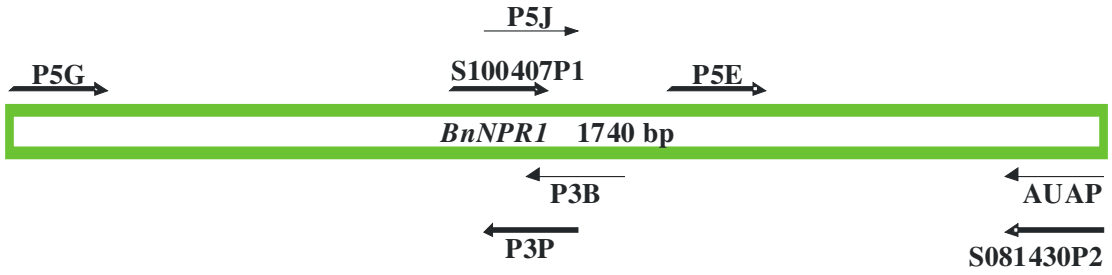
Total RNA was isolated using Trizol (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions and northern analysis was performed as described in Chapter 2. Hybridization probes contained the entire coding regions of *Arabidopsis PR-1* (Uknes et al., 1992), *AtNPR1* or *BnNPR1* that were prepared according to Liu et al. (2005).

4.3. Results

4.3.1. Isolation and functional prediction of the *Brassica napus NPR1* cDNA

The sequence of an Expressed Sequence Tag (EST) from *B. napus* variety DH12075 encoding a protein related to *AtNPR1* was obtained from AAFC, Saskatoon Research Station. Preliminary sequence analysis of the EST indicated that the 3' end did not show any sequence similarity to *AtNPR1*. The EST also lacked a clear poly A+ tail, suggesting that it might represent a pseudo gene or be the result of a rearrangement during the cloning process. Therefore, the 5' end of the EST was used as a guide to amplify a cDNA clone with similarity to *AtNPR1* throughout its length by PCR, including 3' random amplification of cDNA ends (RACE). Initial attempts using cDNA prepared from total RNA of *B. napus* seedlings resulted in the amplification of a 1.1 Kb fragment (primers P5G and P3B; Figure 4.1A). However, these were unsuccessful at isolating the entire coding and the 3' non-coding regions. Given that difficulties in isolating the sequence could be due to the quality of RNA/cDNA or the abundance of the gene in the cDNA pool, attempts were made to circumvent these problems. Five-day-old seedlings

A



P5G + P3B = ~ 1.1 Kb

P5G + P3P = 890 bp

P5J + S081430P2 = 890 bp

P5E + AUAP = ~ 700 bp

S100407P1 + S081430P2 = ~ 1 Kb

B

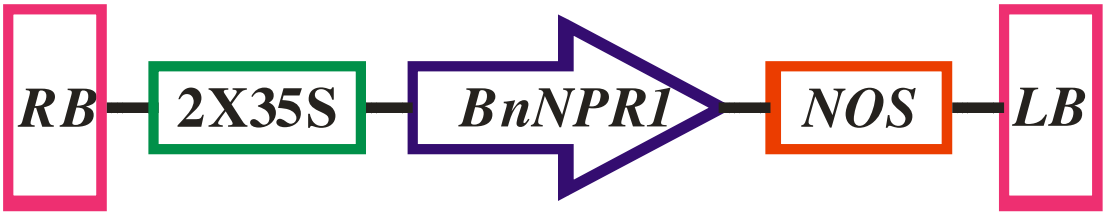


Figure 4.1A. Schematic representation of *BnNPR1* showing the primer positions used in PCR and 3' RACE. The primer combinations and expected band sizes (not to scale) are shown above. **4.1B.** Diagram showing the T-DNA portion of the expression vector (pCAMBIA 2300 derivative) used for *Arabidopsis* and *Brassica napus* transformations. *RB* and *LB*, right and left T-DNA border repeats; 2X35S, double Cauliflower Mosaic Virus 35S promoter with Alfalfa Mosaic Virus translational enhancer; *BnNPR1*, *B. napus NPR1 gene*; *NOS*, nopaline synthase terminator. The diagram is not to scale.

were used to avoid heavy aromatic compounds and phenolics that are found in older leaves. Because the expression of *AtNPR1* increases after SA treatment in *Arabidopsis* (Cao et al., 1997) *B. napus* seedlings were sprayed with 2 mM SA and samples were collected after 18 h. These modifications increased the total RNA yield at least by 10 fold. From this total RNA, mRNA was isolated prior to 3' RACE using various forward primers along with the reverse primer (AUAP) provided in the 3' RACE kit. A 700 bp product was obtained with the P5E forward and AUAP reverse primer combination (Figure 4.1A). Sequence analysis showed that this PCR product contained the 3' end of the gene. However, there was still a very small region between P3B and P5E primers that was not present in either of the PCR products obtained. Using the S100407P1 primer that is upstream of P3B and the S081430P2 primer that includes the putative stop codon, an expected band of 1Kb was obtained (Figure 4.1A). Sequence analysis of this 1 Kb fragment confirmed the presence of the missing sequence from the other two clones.

Once the complete sequence of the putative *BnNPR1* homolog was obtained, the cDNA was reconstructed in two fragments, and ligated together. The full-length coding sequence of the reconstructed gene, called *BnNPR1*, is 1740 bp in length with the potential to encode a protein of 579 aa with an estimated molecular weight of 64.6 kDa (Figure 4.2A). The primary amino acid sequence of *BnNPR1* is 65.9% similar to that of *AtNPR1* (Figure 4.2B). The PROSITE tool was used to identify domains of potential biological significance. It predicted ten different biologically significant domains including one BTB domain (77 aa; 66-142) and two ARD domains (76 aa; 292-367 and 27 aa; 326-352). Similar to AtNPR1, BnNPR1 did not contain sequences characteristic of DNA-binding or transcriptional activation domains. Therefore, they are unlikely to act as a transcription factors by themselves. The BTB/POZ and ARD are known to be involved in protein-protein interactions (Aravind and Koonin., 1999). The predicted BTB/POZ domain of BnNPR1 showed 70 % sequence similarity with the corresponding region of AtNPR1. The ankyrin repeats of AtNPR1 are known to be important for interaction with TGA factors (Després et al., 2000; Zhang et al., 1999). Ankyrin repeats one through four of AtNPR1 and BnNPR1displayed 72.7 %, 69.7 %, 72.7 % and 52.9 % sequence similarity, respectively. Overall, these sequence alignments suggest that the function of these protein-protein interaction domains is well-conserved between AtNPR1 and

A

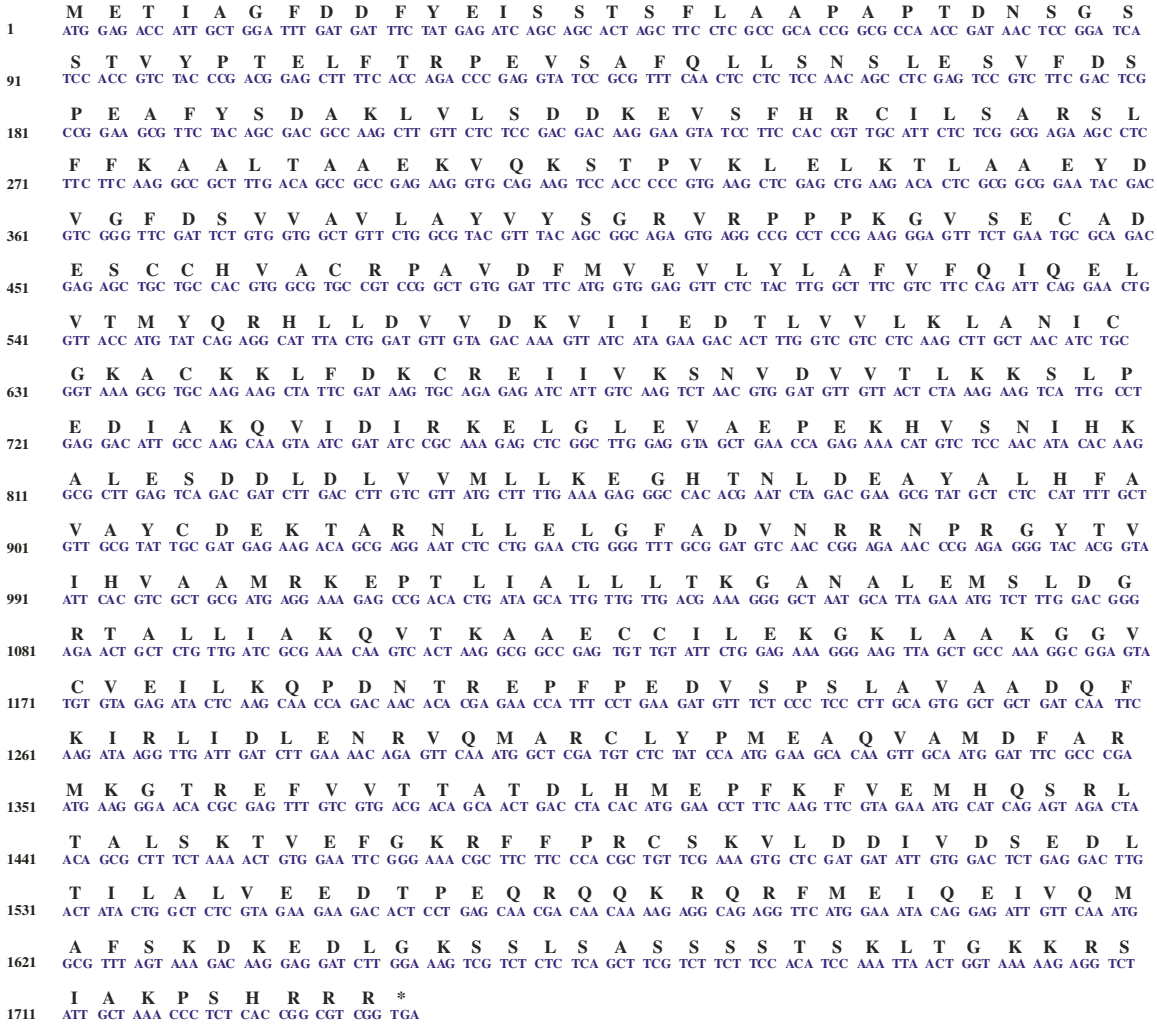


Figure 4.2A. Nucleotide (in blue) and deduced amino acid sequence (in black) of the *Brassica napus NPR1* coding sequence. The deduced protein sequence is shown above the corresponding DNA sequence.

B

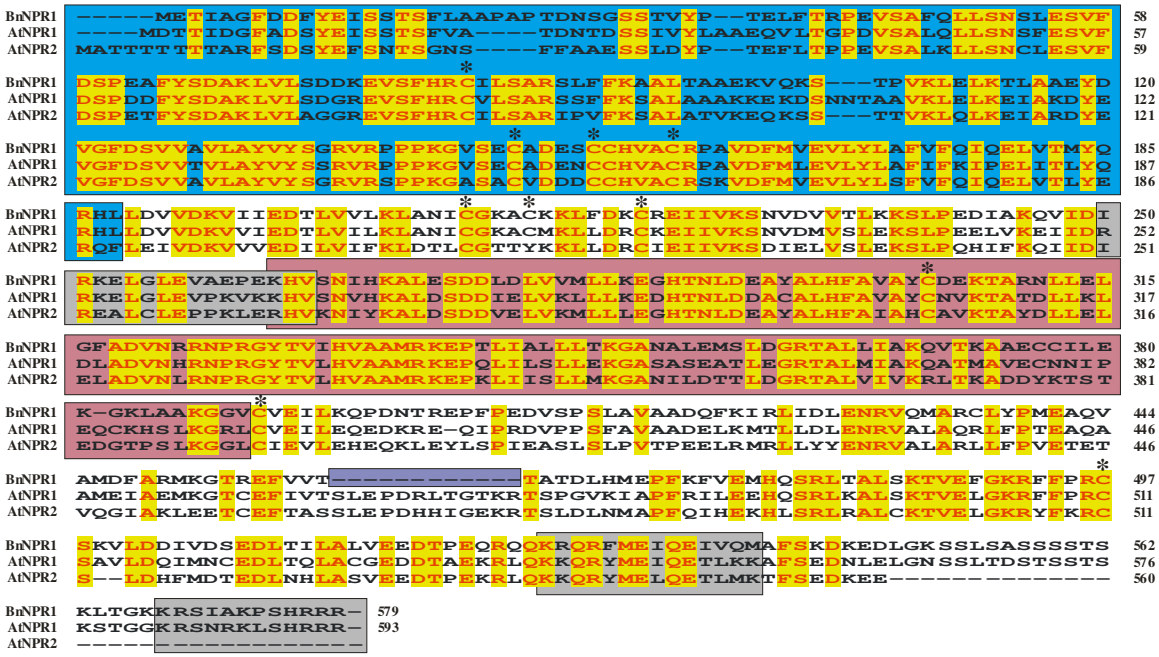


Figure 4.2B. Comparison of the deduced amino acid sequences of the predicted *Brassica napus* NPR1 (BnNPR1) and *Arabidopsis* NPR1 (AtNPR1) and NPR2 (AtNPR2) proteins. Boxed regions indicate BTB/POZ (blue, 1-190), ARD (pink, 265-297; 294-326; 328-360; 361-393) and NLS (grey, 252-265; 541-554; 582-593) domains as identified in AtNPR1. The purple box in the C-terminal of BnNPR1 represents the 12 amino acid deletion corresponding to SLEPDRLTGTKR in AtNPR1. All the 10 cysteines (*) that are conserved among NPR1 related proteins are also conserved in BnNPR1. Identical amino acids are represented in red with a yellow background and dashes indicate gaps introduced to maximize alignment.

BnNPR1. Studies indicate that two of the three putative nuclear localization signals (NLS) that are present in the C-terminal end of AtNPR1 are involved in targeting AtNPR1 protein to the nucleus (Kinkema et al., 2000). Alignment of these NLSs of AtNPR1 to the predicted NLSs of BnNPR1 displayed a 64.3 %, 57.1 % and 75 % homology, respectively, indicating that their function may be conserved in BnNPR1.

In *Arabidopsis*, it has been shown that in the uninduced state, AtNPR1 is present in the form of an oligomer that is formed through intermolecular disulfide bonds (Mou et al., 2003). All the 10 conserved cysteines that are present in AtNPR1 (Figure 4.3A; C82, 150, 155, 160, 212, 216, 223, 306, 394 and 511) are also present in BnNPR1 suggesting that similar to AtNPR1, BnNPR1 protein has the potential to form disulfide bonds and may be regulated through redox-mediated signaling. There are seven other cysteines in AtNPR1 (C156, 297, 378, 385, 457, 521 and 529) and only two of them (C156 and 378) are conserved in BnNPR1. There are two additional cysteines (C377 and 436) in BnNPR1 that do not match with any of the cysteines of the AtNPR1 protein sequence.

The most salient difference between AtNPR1 and BnNPR1 is a 12 aa deletion in the C-terminal end of BnNPR1 that corresponds to aa 462-473 of AtNPR1 (SLEPDRLTGTKR). Analysis of this sequence using the protein data base PROSITE indicated a probable casein kinase II phosphorylation site but did not give any similarity score. This 12 aa deletion in BnNPR1 does not contain any of the putative NLSs.

The *Arabidopsis* genome contains 5 other *NPR1*-related genes (*AtNPR2* through *AtNPR6*; Arabidopsis Genome Initiative, 2000; Liu et al., 2005). Of these, the protein encoded by *AtNPR2* is most closely related to AtNPR1. Preliminary results from the Fobert lab (unpublished) indicate that *AtNPR2* is also involved in controlling *PR* gene expression and possibly SAR in *Arabidopsis*. Sequence alignment of BnNPR1 with AtNPR2 displayed a similarity of 56%, compared to 65.5% observed in the case of AtNPR1 (Figure 4.2B). This indicates that the function of BnNPR1 is likely to be more similar to AtNPR1 than AtNPR2. A phylogenetic tree of proteins annotated as being orthologs of AtNPR1 was constructed using the Clustal W feature of the Megalign program (DNAStar, Lasergene Inc., Figure 4.3B). BnNPR1 was found to be most closely related to a NPR1 sequence from *B. juncea* (BjNPR1; 97.6 %). *B. juncea* and *B. napus* are both allotetraploid species and share a common ancestor (“A” genome; see Chapter

Figure 4.3A. Alignment of the predicted *Brassica napus* NPR1 protein sequence to NPR1 orthologs from *Brassica juncea*, *Arabidopsis thaliana*, *Nicotiana tabacum* (tobacco), *Lycopersicum esculentum* (tomato) and *Oryza sativa* (rice) generated using Multiple Alignment Algorithm of the Vector NTI program. Amino acids that are identical in all sequences are represented in red with a yellow background and dashes indicate gaps introduced to maximize alignment.

B

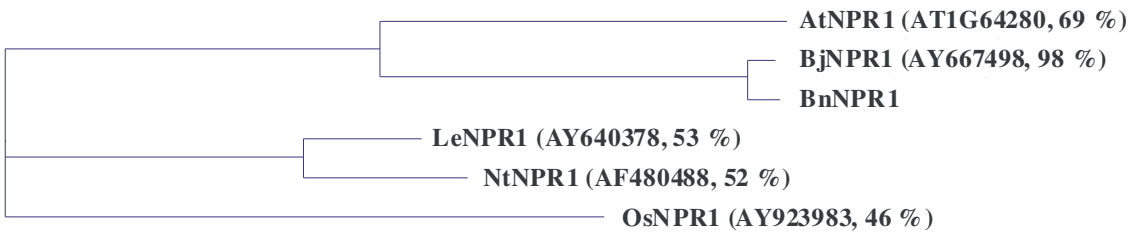


Figure 4.3B. Phylogenetic tree of NPR1 orthologs from *Brassica napus, Brassica juncea*, *Arabidopsis thaliana*, *Nicotiana tabacum* (tobacco), *Lycopersicum esculentum* (tomato) and *Oryza sativa* (rice) generated using the Guide Tree Algorithm of the Vector NTI program. The locus identification number of AtNPR1 and Genbank accession numbers of all other proteins, as well as the amino acid identity of these proteins to *Brassica napus* NPR1 in percentage (%) are indicated in brackets.

1.8). It is therefore not surprising that *NPR1* genes from these two species are highly related. Substantial similarity was also observed between BnNPR1 and NPR1 homologs from other species such as *Nicotiana tabacum* (tobacco; 50.7 %), *Lycopersicum esculentum* (tomato, 50.5 %) and *Oryza sativa* (rice, 44.5 %) indicating that the NPR1 sequence is well conserved amongst mono- and dicotyledonous plants. An alignment of the amino acid sequences of the above proteins is illustrated in Figure 4.3A.

4.3.2. Expression pattern of *BnNPR1* in response to pathogen challenge and SAR induction

To determine the expression pattern of *BnNPR1,* northern blot hybridization was performed using leaves from *B. napus* at different times following challenge with avirulent *Psm* 1120B (10^6 cfu ml^{-1}) or treatment with BTH (37.5 µg a.i. ml^{-1}). The *BnNPR1* transcript could not be detected in untreated tissue (Figure 4.4). However, transcript was detectable in infected and BTH treated leaves one day following treatment and increasing slightly 1-week after treatment. Thereafter, levels in infected tissues declined, while those in BTH-treated leaves remained high even after three-weeks. *BnNPR1* transcripts were not detected in non-infected, systemic leaves until 1-week following infection with *P. syringae*. Levels of *BnNPR1* transcript detected in systemic leaves at this time point or later were very low. These results indicate that *BnNPR1* is responsive to pathogen infection and BTH, but that the induction kinetics are weak and slow.

4.3.3. *BnNPR1* cDNA partially complements *Arabidopsis npr1* mutations

It was hypothesized that if *BnNPR1* is the *B. napus* ortholog of *AtNPR1*, it should be capable of complementing *Arabidopsis npr1* mutations. To this end, the *BnNPR1* coding region was fused to the CaMV35S promoter and introduced into the *npr1-2* and *npr1-3* genetic backgrounds by *Agrobacterium*-mediated transformation. T_3 (Figures 4.5A and 4.5B) and T_2 (Figures 4.6A and 4.6B) generation lines, expressing low, medium and high levels of *BnNPR1* were chosen for further study.

The *npr1-3* and *npr1-2* mutations were both generated by chemical mutagenesis (Glazebrook et al., 1996) and the resulting lesions are described in the introduction to this Chapter (section 5.1). One phenotypic consequence of these mutations is that plants do not express SA-inducible *PR* genes following exposure to this metabolite (Cao et al.,

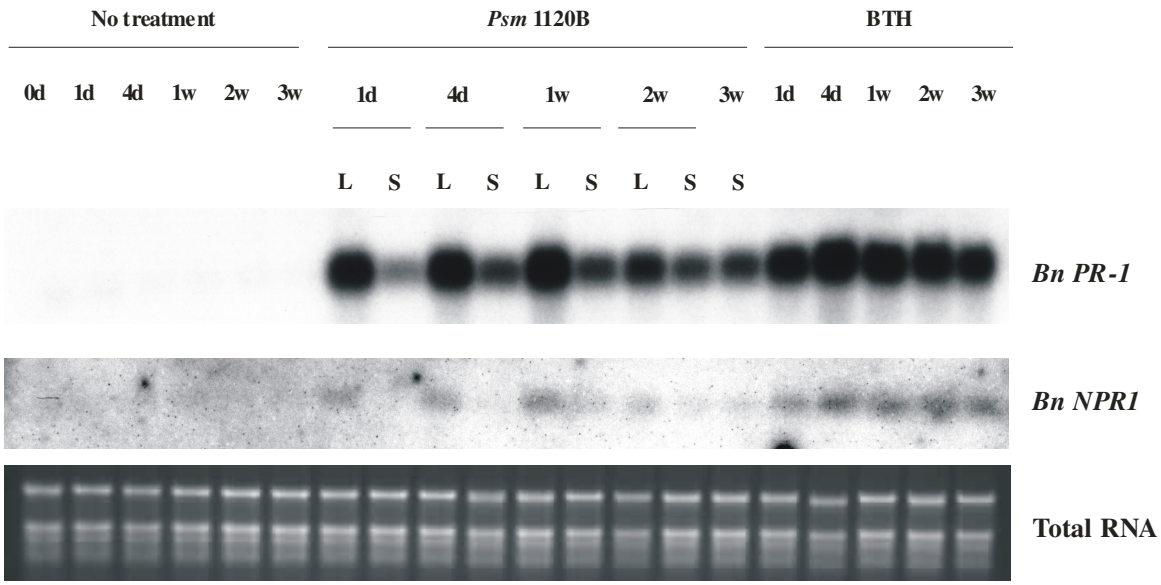


Figure 4.4. Expression of *BnNPR1* in untransformed *Brassica napus* plants after induction of SAR either by infiltration of avirulent *Pseudomonas syringae* pv. *maculicola* 1120B or spraying with BTH. Five µg of total RNA were extracted from leaves at the times indicated, blotted on a nylon membrane and hybridized with radioactively labeled, full-length *BnPR1*. The blot was stripped and reprobed using the full-length *BnNPR1* cDNA. Photo of ethidium bromide-stained gel is included as a measure of RNA loading.

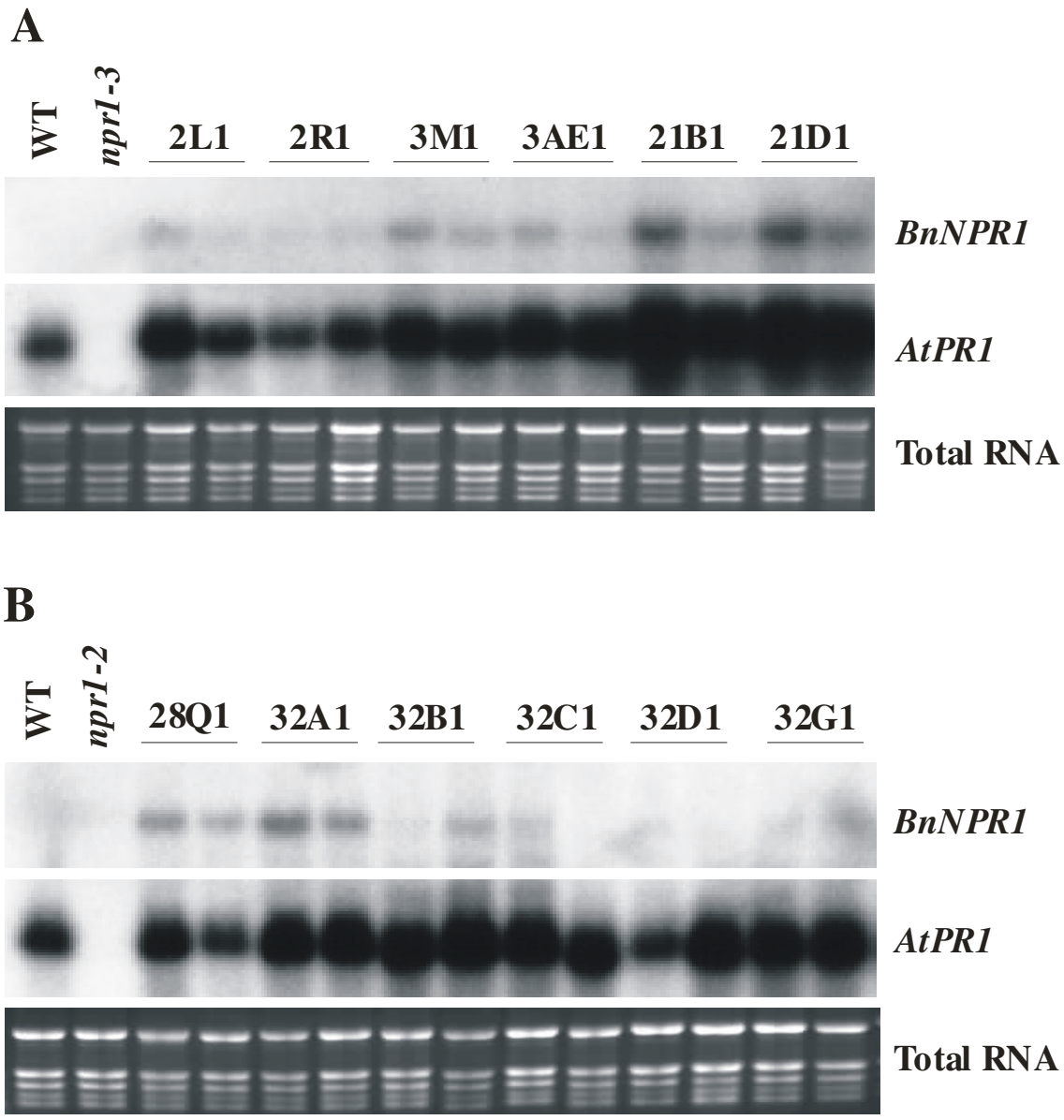
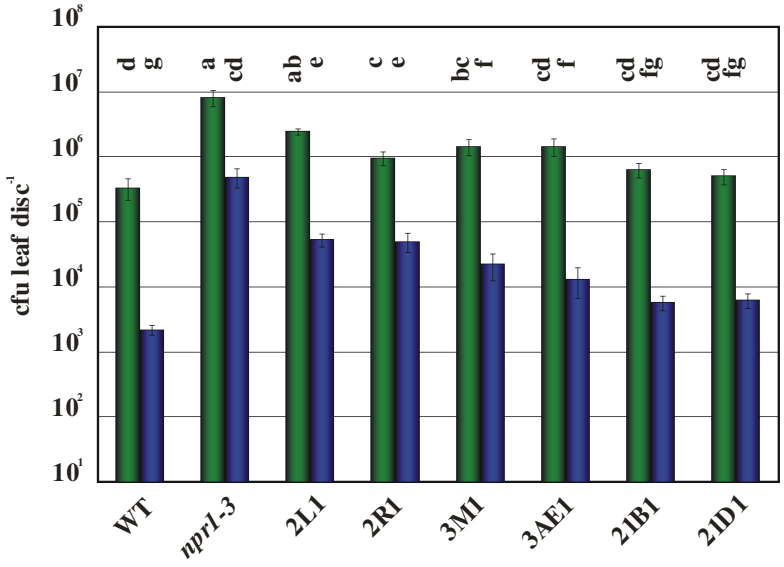


Figure 4.5A and 4.5B. Expression of salicylic acid (SA)-activated *AtPR-1* gene in *npr1-3* (A) and *npr1-2* (B) transgenic plants (T_3 generation) transformed with the CaMV35S-*BnNPR1* construct following treatment with SA. Two different plants from each T_3 line were analyzed. Northern blot analyses were performed as described in Figure 4.4A. Plants from the same transgenic event are labeled starting with the same number. Three independent events (lines #2, 3 and 21) were tested in (A) and two independent events (#28 and 32) in (B). Photos of ethidium bromide-stained gels are included as a measure of RNA loading.

C



D

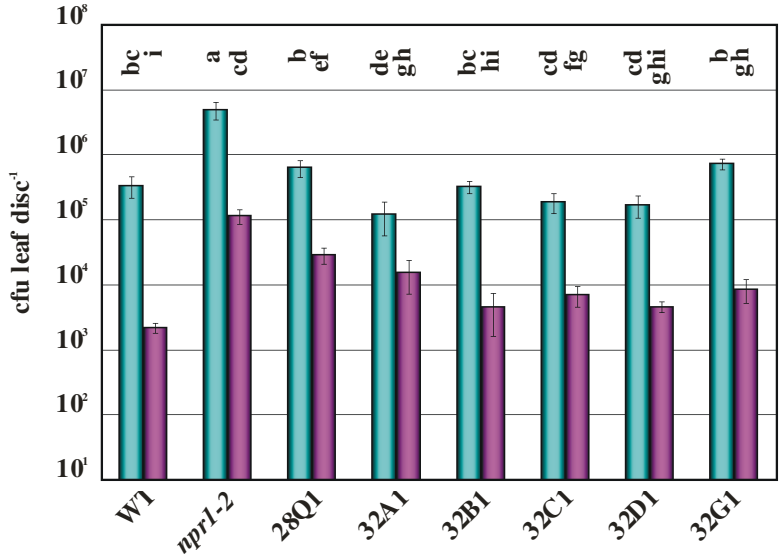


Figure 4.5C and 4.5D. Growth of *Pseudomonas syringae* pv. *tomato* DC3000 in *npr1-3* (C) and *npr1-2* (D) transgenic plants (T_3 generation) expressing *BnNPR1* following treatment with water (green and sky blue bars) or SA (dark blue and pink bars). Plants were treated with 0.5 mM SA prior to infection with virulent *P. syringae* (10^5 cfu ml^{-1}). Bacterial counts (cfu leaf $disc^{-1}$) were determined three days after infection. Each sample consisted of 8 leaf discs from one single plant and every data point represents the mean ± SE of 6 samples. Letters above the bars indicate treatments that are statistically the same by ANOVA analysis of log-transformed means.

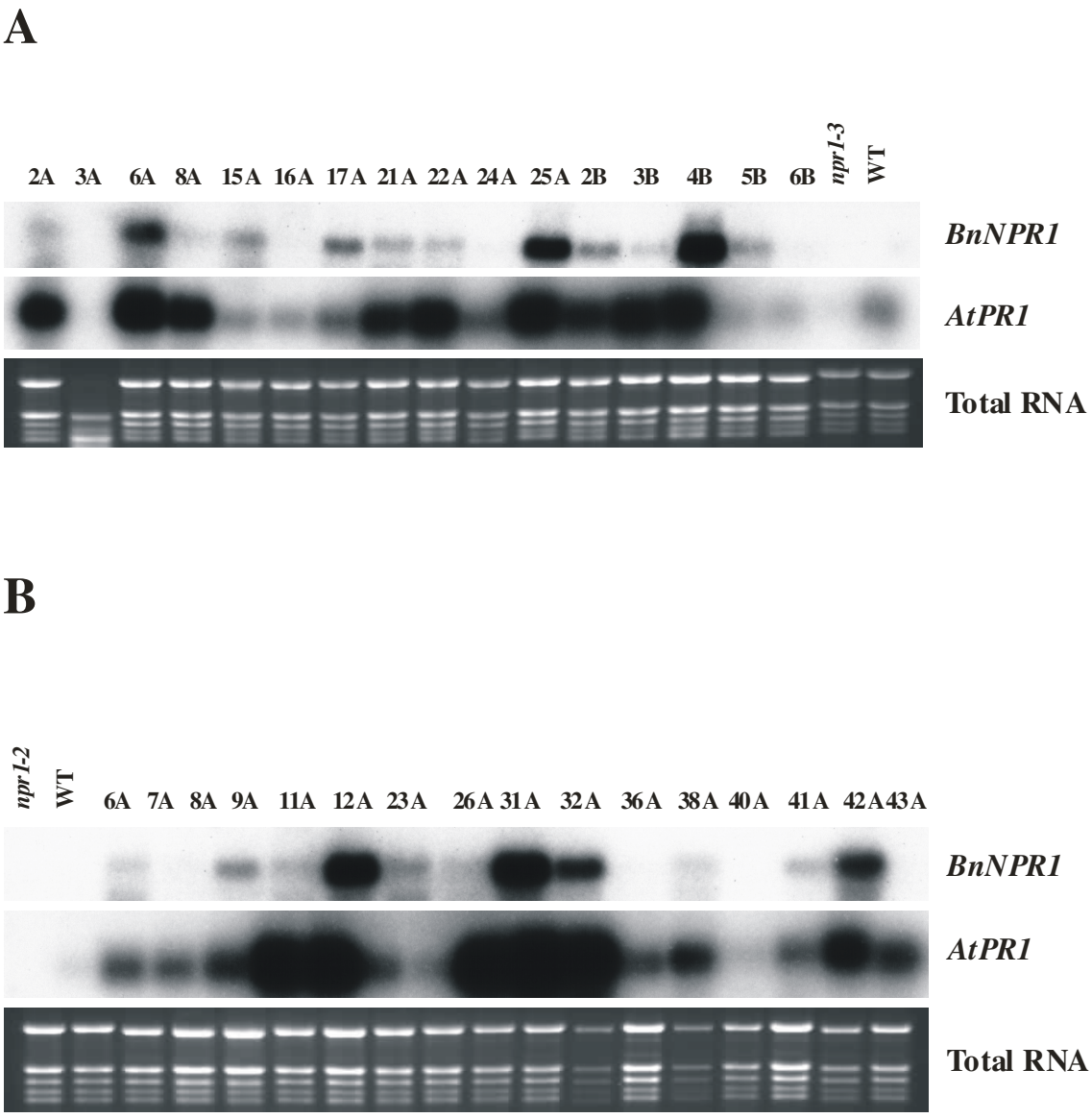
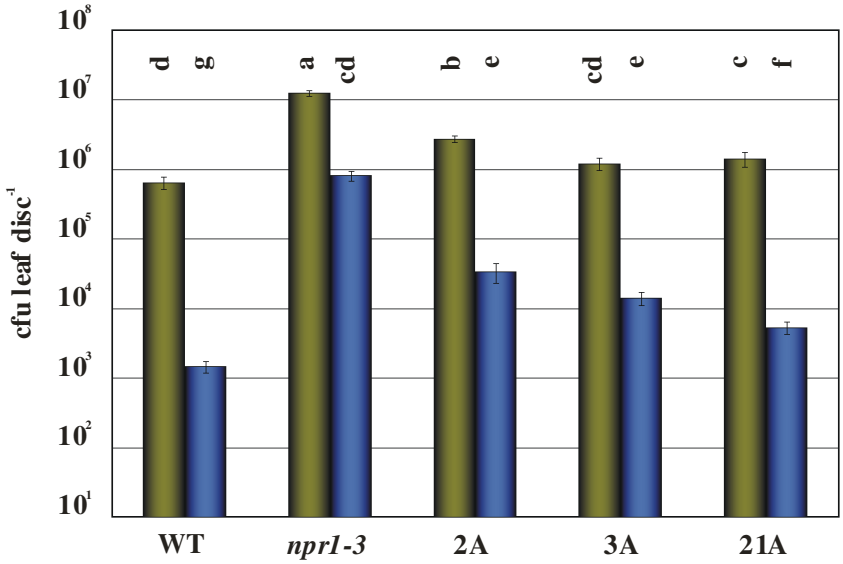


Figure 4.6A and 4.6B. Expression of salicylic acid (SA)-activated *AtPR-1* gene in *npr1-3* (A) and *npr1-2* (B) transgenic plants (T_2 generation) expressing *BnNPR1* following treatment with SA. Northern blot analyses were performed as described in Figure 4.4A. Plants from the same transgenic event are labeled starting with the same number. Photos of ethidium bromide-stained gels are included as a measure of RNA loading.

C



D

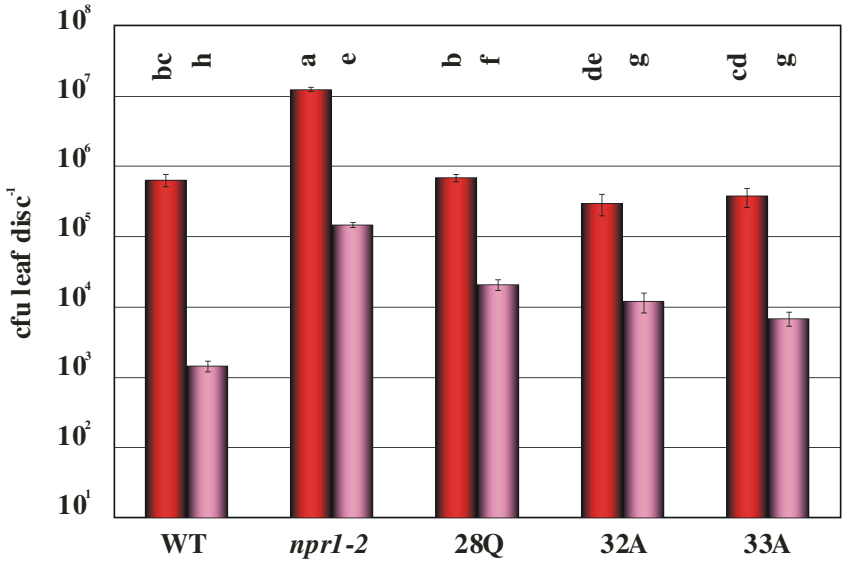


Figure 4.6C and 4.6D. Growth of *Pseudomonas syringae* pv. *tomato* DC3000 in *npr1-3* and *npr1-2* transgenic plants (T_2 generation) expressing *BnNPR1* following treatment with water (green and red bars) or SA (blue and pink bars). Plants were treated with 0.5 mM SA prior to infection with virulent *P. syringae* (10^5 cfu ml^{-1}). Bacterial counts (cfu leaf $disc^{-1}$) were determined three days after infection. Each sample consisted of 8 leaf discs from one single plant and every data point represents the mean ± SE of 6 samples. Letters above the bars indicate treatments that are statistically the same by ANOVA analysis of log-transformed means.

1997; Liu et al., 2005). In order to evaluate the ability of *BnNPR1* to complement *PR* gene expression, wild-type, *npr1*, and six independent T_3 lines of transgenic *npr1* plants expressing *BnNPR1* were sprayed with 0.5 mM SA. RNA was extracted from leaf tissue collected 18 h later and analyzed for *PR-1* expression by northern blot hybridization (Figure 4.5A and 4.5B). Under these conditions, the wild-type plants expressed *PR-1* whereas the *npr1-3* and *npr1-2* mutants did not, as anticipated (Figures 4.5A and 4.5B). In contrast, transgenic *npr1* lines expressing *BnNPR1* expressed *PR-1* at levels equal to, or higher than, those observed in wild-type plants. These results demonstrate that *BnNPR1* is able to functionally complement this aspect of the *npr1-3* and *npr1-2* mutations in *Arabidopsis*.

In addition to compromised *PR* gene expression, *npr1* mutant plants display enhanced susceptibility to virulent pathogens (Glazebrook et al., 1996). To test whether *BnNPR1* can complement this aspect of the mutant phenotype, *npr1-3* and *npr1-2* plants expressing *BnNPR1* (T_3 plants) were sprayed with water or SA three days prior to inoculation with 10^5 cfu ml^{-1} of virulent *Pst* DC3000. Three days post-inoculation disease resistance was assessed by quantifying viable bacteria in infected leaves (Figure 4.5C and 4.5D). The average colony forming units (cfu) per leaf disc were calculated and log-transformed data were analyzed statistically by Analysis of Variance (ANOVA), General Linear Model, as implemented in the SAS software package (SAS Institute Inc., Cary, NC, USA). When compared to the wild-type, *npr1-3* and *npr1-2* plants displayed a 24.4 and 14.5-fold increase in bacterial growth, respectively. These results were statistically significant as determined by *t*-test within ANOVA, confirming the enhanced disease susceptibility phenotype of these *npr1* mutants. The transgenic *npr1-3* plants expressing the *BnNPR1* gene displayed a 3.3 to 16-fold reduction in bacterial growth (Figure 4.5C) and *npr1-2* plants expressing the *BnNPR1* gene displayed a 6.7 to 40-fold reduction in bacterial growth compared to the respective non-transformed parents (Figure 4.5D). These differences were found to be statistically significant at $p \leq 0.05$ using *t*-test within ANOVA. However, all the transgenic lines in the *npr1-3* background and two lines in *npr1-2* background (28Q1 and 32G1) continued to allow more bacterial growth than the wild-type plants ($p \leq 0.05$). Together, these results indicate that *BnNPR1* is able to

partially complement the disease resistance defect observed in the *npr1-3* and *npr1-2* mutant plants.

Although not specifically reported for the *npr1-2* and *npr1-3* alleles, *npr* mutant plants typically continue to be more susceptible than the wild-type after exposure to SAR-inductive treatments (Cao et al., 1994; Delaney et al., 1995). The ability of transgenic plants expressing *BnNPR1* to respond to a SAR-inductive pre-treatment was tested by spraying plants with SA three days prior to inoculation with *Pst* DC3000. When compared to the mock pre-treatment (water), spraying with SA resulted in a large reduction (154-fold) of bacterial growth in wild-type plants (Figure 4.5C and 4.5D). These results were statistically significant using ANOVA. More modest growth reductions that were also statistically significant were observed in the *npr1-3* and *npr1-2* mutants (17- and 43-fold, respectively). Thus, under the experimental conditions used in this study, SA pre-treatment conferred additional disease resistance in these mutant genotypes. However, bacterial titres remained significantly higher than those observed in wild-type following SA pre-treatment.

SA pre-treatment was also effective at reducing bacterial growth in all the transgenic lines tested (Figure 4.5C and 4.5D). When compared to plants from the same lines that did not receive SA treatment, *npr1-3* mutants expressing *BnNPR1* displayed a 20-110 fold reduction in bacterial growth after SA treatment while *npr1-2* mutants expressing *BnNPR1* displayed a 7-85 fold reduction. These results were found to be statistically significant ($p \leq 0.05$). Thus, although substantial reductions in bacterial growth were observed in both the mutant plants expressing *BnNPR1* after SA treatment, they did not reach the same levels as observed in wild-type plants that received the same pre-treatments. Four out of six transgenic lines in both the *npr1-3* (2L1, 2R1, 3M1 and 3AE1; Figure 4.5C) and *npr1-2* backgrounds (28Q1, 32A1, 32C1 and 32G1; Figure 4.5D) fully complemented the disease phenotype. This suggests that *BnNPR1* is able to function in a similar way as the native *AtNPR1* but not to the same extent, once again suggesting that *BnNPR1* is able to partially complement the *npr1* mutation. Similar results were obtained in T_2 plants as shown in Figure 4.6. Thus, the phenotypic consequences of expressing *BnNPR1* in transgenic *Arabidopsis* were heritable. Overall, there was no correlation between the levels of *BnNPR1* transcript, levels of *PR-1*

transcript, and the amount of disease resistance observed in either the T_2 or T_3 generations.

4.3.4. Overexpression of *AtNPR1* and *BnNPR1* in *Brassica napus* provides enhanced resistance against *Pseudomonas syringae* pv. *maculicola*

As detailed in Section 4.1, expression of *AtNPR1* and its rice ortholog can lead to enhanced resistance to disease. To determine if expression of *AtNPR1* and overexpression of *BnNPR1* conferred enhanced resistance to disease in *B. napus*, transgenic lines containing each gene separately were generated using *Agrobacterium*-mediated transformation. Northern blot hybridization confirmed that the plants recovered expressed different steady-state levels of *AtNPR1* (Figure 4.7A) or *BnNPR1* (Figure 4.7B). There were no obvious developmental aberrations observed in any of these plants. Four different independent transgenic lines expressing *AtNPR1* (6B, 12, 18 and 21A) and six independent transgenic lines expressing *BnNPR1* (2A, 3A, 4, 5, 7A and 9) along with non-transformed controls were analyzed in this study. Third and fourth leaves of three-week-old-plants were inoculated with 10^5 cfu ml^{-1} of virulent *Psm* 1848B and disease resistance was assessed by quantifying viable bacteria four days post-infection. Compared to untransformed controls, transgenic lines expressing *AtNPR1* and *BnNPR1* displayed a 3-9 fold and 6-86 fold reduction in bacterial growth, respectively (Figure 4.7C). These differences were found to be statistically significant at $p \leq 0.05$, indicating that expression of either *AtNPR1* or *BnNPR1* in *B. napus* is capable of enhancing disease resistance against virulent *Psm*. Bacterial titres in three of the transgenic lines expressing *AtNPR1* (6B, 18, 21A) were not significantly different from those in lines 5 and 7A expressing *BnNPR1*. Bacterial titres in two of the lines expressing *BnNPR1* (2A and 3A) were found to be statistically lower than all other transgenic lines analyzed. There was no correlation between transgene expression (*AtNPR1 or BnNPR1*) and levels of resistance achieved. Also, the transgenic *B. napus* plants expressing either *AtNPR1 or BnNPR1* did not display constitutive *BnPR* gene expression (results not shown). Levels of *PR* gene expression in these plants following SAR pre-treatments remain to be determined.

4.4. Discussion

This study reports on the isolation and functional characterization of a putative *NPR1* ortholog from *B. napus*. Starting from an EST clone that showed similarity to the

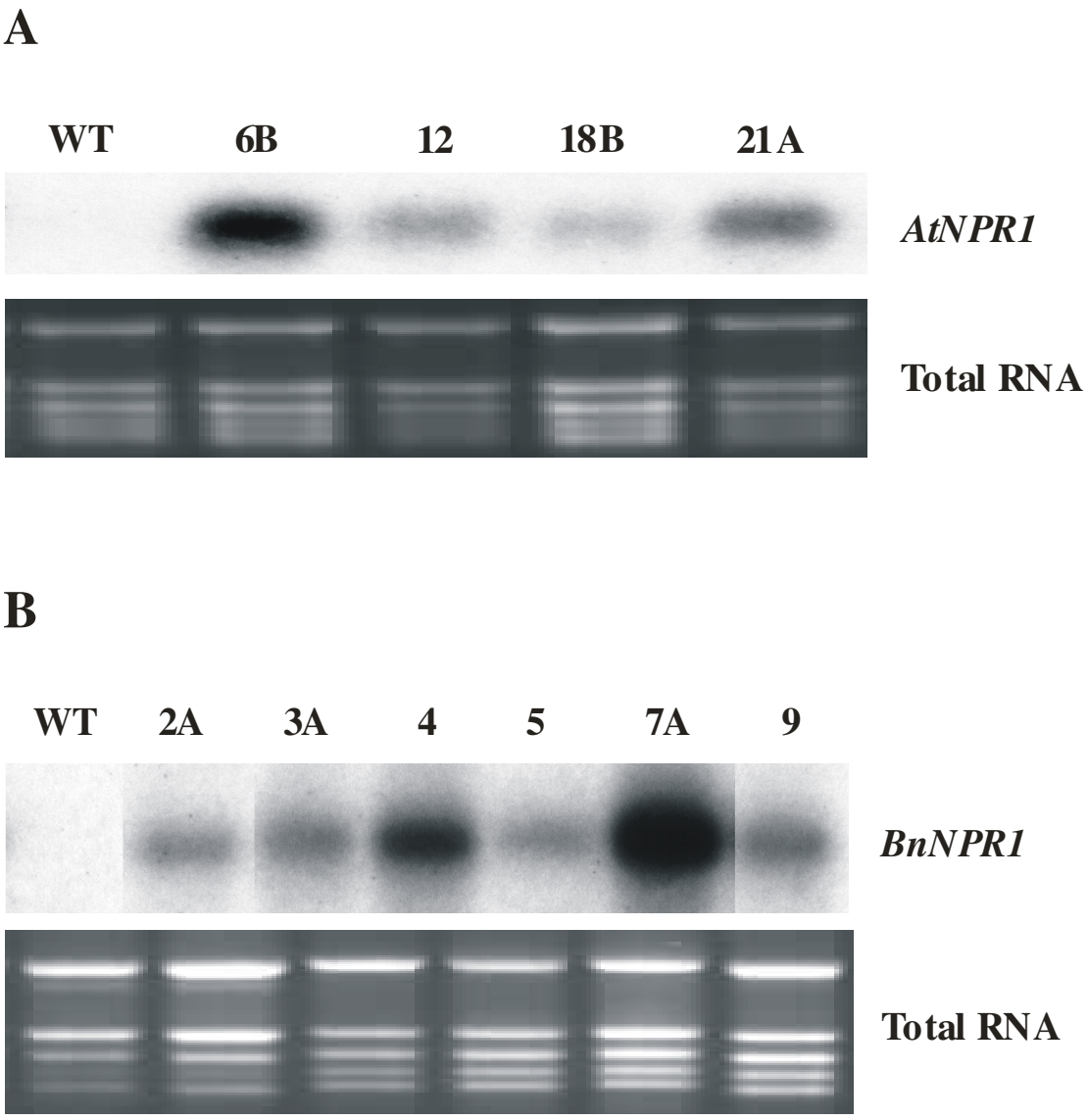


Figure 4.7A and 4.7B. Northern blot analysis of T_0 transgenic *Brassica napus* plants expressing *AtNPR1* (A) and *BnNPR1* (B) genes under the control of the CaMV35S promoter. Total RNA was isolated from 4-week-old plants and five µg was separated on a gel before transferring to a nylon membrane. Hybridization was performed using radioactively labeled full-length *AtNPR1* and *BnNPR1* probes as indicated. Photographs of ethidium bromide-stained gels are included as a measure of RNA loading.

C

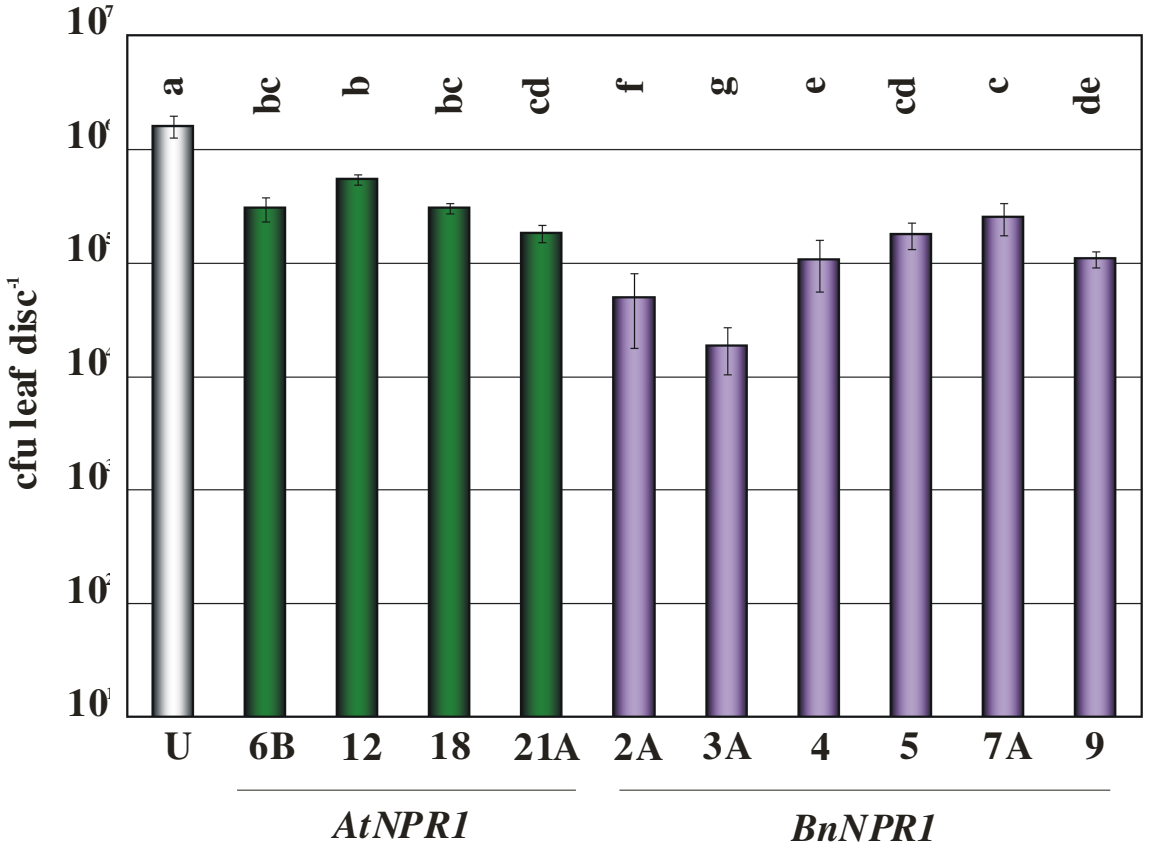


Figure 4.7C. Growth of *Pseudomonas syringae* pv. *maculicola* in transgenic *Brassica napus* plants expressing *AtNPR1* and *BnNPR1* genes. Three-week-old T_1 transgenic plants were inoculated with virulent *P. syringae* pv. *maculicola* 1848B at 10^5 cfu ml^{-1}. Bacterial growth was quantified four days after inoculation using serial dilution. Each sample consisted of 8 leaf discs from one single plant and every data point represents the mean ± SE of 6 samples representing 6 individual untransformed (U) and transgenic plants. Letters above the bars indicate treatments that are statistically the same by ANOVA analysis of log-transformed means. Experiment using transgenic *B. napus* plants expressing *AtNPR1* was repeated once with similar results.

AtNPR1 gene, a cDNA containing a full length coding region was isolated from *B. napus* seedlings. This cDNA is predicted to encode a protein with close to 66% similarity to AtNPR1. This level of similarity was found to be even higher within structural elements, including protein-protein interaction domains (BTB/POZ and ARD) and NLSs, known to be required for AtNPR1 function (Cao et al., 1997; Ryals et al., 1997; Després et al., 2000; Zhang et al., 1999; Kinkema et al., 2000; Rochon, Fobert, Després et al. unpublished data). Furthermore, all the 10 cysteines (including C82 and C216) found to be conserved among known NPR1-like proteins (Mou et al., 2003) are also predicted to be conserved in the product of the putative *B. napus* ortholog. Together, analysis of the primary sequence indicates that NPR1 function and regulation have been highly conserved between *B. napus* and *Arabidopsis*. This notion is further substantiated by complementation tests demonstrating that BnNPR1 can functionally substitute for AtNPR1 in certain mutant genetic backgrounds (see below). Thus, although the biochemical properties of BnNPR1 have not been directly studied, one would speculate, based on my results, that nuclear localization of this protein may be regulated by redox regulation of conserved cysteine residues following SAR induction and require the C-terminal NLSs. Nuclear BnNPR1 is likely to interact with TGA factors through its ARD, to stimulate their DNA-binding properties and regulate *BnPR* gene expression.

The genus *Brassica* and *Arabidopsis* both belong to the family Brassicaceae (Cruciferae) and are predicted to have diverged 16-19 million years ago (Lenoir et al., 1997). However, protein encoding regions and gene regulatory regions of their genomes have been well conserved to maintain the function of the proteins (Parkin et al., 2005). Therefore, it is quite possible to see similarity between *Arabidopsis* and *B. napus* protein functional domains.

The only striking difference between NPR1 proteins from *Brassica* and all other sequences that were compared is a 12 amino acid deletion in the C-terminal end of the former. In addition to BnNPR1, this deletion was also present in an NPR1 homolog from *B. juncea*, which is a close relative of *B. napus*. Studies on genome evolution of *Brassica* indicate that *B. napus* and *B. juncea* are two different amphidiploids which share *B. rapa* as a common ancestor. Therefore, it is not very surprising to see high degree of similarity between these two species. The functional significance of the 12 amino acid deletion in

NPR1 proteins from Brassica is unknown; however, the construct was still able to complement the Arabidopsis *npr1* mutants.

Overall, the expression patterns of *AtNPR1* and *BnNPR1* are very similar. However, important differences were also apparent. The expression of both genes is increased following pathogen infection or treatment with SAR-inducing chemicals (Figures 4.4; Liu et al., 2005; Ryals et al., 1997). However, whereas increased expression of *AtNPR1* was observed to be rapid, occurring within hours (e.g. Liu et al., 2005) only low levels of *BnNPR1* could be detected after one day following inoculation with *P. syringae* or treatment with BTH. Data presented in Chapter 2 showed that BTH-induced SAR in *B. napus* is much more effective in providing resistance against the bacterial pathogen *Psm* and the fungal pathogen *L. maculans* when compared to pre-inoculation with HR-inducing *Psm*. The capacity of BTH to activate *BnNPR1* faster and to higher levels might explain why chemical treatment is much more effective in inducing a stronger SAR response.

The *npr1* mutant plants are compromised in the expression of *PR* genes such as *PR-1*, *PR-2* and *PR-5* even after the induction of SAR and exhibit enhanced susceptibility to pathogens (Cao et al., 1994). Using two different lines of evidence I have shown that the *BnNPR1* is able to complement both these phenotypes when expressed in *npr1* mutant plants. *PR-1* transcript levels in several of the transgenic lines were similar to or higher than those observed in wild-type plants, suggesting that the *BnNPR1* is able to fully substitute for the *Arabidopsis* ortholog. Second, the enhanced susceptibility phenotype of *npr1* mutant plants was also complemented in plants that were expressing *BnNPR1*. Although bacterial growth measured in transgenic *npr1-3* and *npr1-2* lines expressing *BnNPR1* was significantly lower (except line 2L1 in Figure 4.5C) than that observed in the untransformed mutant parents, it remained higher than that measured in wild-type plants. This indicates that the *BnNPR1* was capable of only partially complementing the enhanced susceptibility phenotype of *Arabidopsis npr1* mutants under the conditions tested. This may be attributed to functional divergence between BnNPR1 and AtNPR1, or to small differences in the associated signaling pathways. In support of this hypothesis, it is noteworthy that BnNPR1 shows 66% similarity, or 44% divergence, to AtNPR1. However, efforts to complement *npr1* mutants using CaMV35S-*AtNPR1* have also

resulted primarily in partial rescue of the disease resistance phenotype (Fobert, unpublished results) indicating that full complementation of this phenotype is difficult to achieve, even with the endogenous protein.

It is noteworthy that the levels of resistance achieved in *npr1-3* and *npr1-2* lines expressing *BnNPR1* did not correlate well with the transcript levels of *PR-1* or *BnNPR1* measured in these lines. Also, there was no correlation between *BnPR1* and *BnNPR1* transcript levels. This suggests that the function of BnNPR1 may be regulated at the post-transcriptional level or that, as suggested by others, resistance depends on production of NPR1 above a critical threshold (Lin et al., 2004). Analysis of BnNPR1 protein levels in the transgenic plants by western blot hybridization would help in resolving whether post-transcriptional control mechanisms are responsible for the poor correlation between disease resistance and transgene expression.

I also studied the effect of overexpression of two *NPR1* homologs (*AtNPR1* and *BnNPR1*) in *B. napus*. The overexpression of *AtNPR1* and *BnNPR1* in *B. napus* were both able to provide enhanced disease resistance against *Psm*. This suggests that *AtNPR1* may be functioning similar to *BnNPR1* in *B. napus* plants. It remains to be determined whether activation of SAR, in combination with the overexpression of *AtNPR1* or *BnNPR1*, is more effective at enhancing disease resistance in *B. napus*.

CHAPTER 5. General Discussion

Systemic acquired resistance was first described in 1961 by Ross and since then this phenomenon has been widely researched in many model plants (Durrant and Dong, 2004., Hammerschmidt and Becker, 1997, Ross, 1961). In my thesis I tried to gain insight into the molecular mechanisms of SAR in the crop plant *B. napus*. I have shown that, in *B. napus*, SAR can be induced by the chemical BTH resulting in significant enhanced resistance against the bacterial pathogen *Psm* and fungal pathogen *L. maculans* (Chapter 2). Biologically induced SAR showed modest levels of enhanced disease resistance against both these pathogens.

A characteristic feature of SAR is the expression of PR genes and proteins (Ryals et al., 1996). Both SAR pre-treatments in my study resulted in the accumulation of *BnPR* gene transcripts. This correlation suggests that the resulting PR proteins may be contributing towards the observed increase in disease resistance. The expression of *BnPR* genes was higher in plants treated with BTH than those pre-inoculated with avirulent *Psm*. The weaker response of biologically induced SAR by *Psm* may be due to the production of either a weak SAR signal and/or the slower transmission of this signal when compared to chemical induction by BTH. This may be one of the reasons for stronger expression of *BnPR* genes leading to an effective SAR response during chemical SAR. By providing a single application of either SAR-inducing pre-treatment 3 weeks prior to virulent *Psm* inoculation, I was able to demonstrate that SAR in *B. napus* can be long lasting which is another important feature of SAR.

Using transgenic plants expressing the bacterial *NahG* gene, it was demonstrated that SA is required for the expression of *PR* genes and involved in the regulation of SAR (Delaney et al., 1994; Gaffney et al., 1993). Similarly, I showed that transgenic *B. napus* plants expressing *NahG* were compromised in SA accumulation leading to ineffective biological SAR. Therefore, my results suggest that SA accumulation is important for SAR in *B. napus*.

In *Arabidopsis* plants that are subjected to biological or chemical SAR pre-treatments, the expression of *PR-1* correlates with that of 31 other genes defined as the *PR-1* regulon (Maleck et al., 2000). This typical defense gene expression pattern was lost in *Arabidopsis* plants expressing the *NahG* gene (Delaney et al., 1994; Gaffney et al., 1993; Maleck et al., 2000). In *B. napus* plants expressing the *NahG* gene, *PR* gene expression was delayed and reduced when compared to wild-type plants. The fact that *BnPR* gene expression was reduced and not completely abolished suggests that there might be other SA-independent pathways in *B. napus* leading to *BnPR* gene expression. Such pathways either exist or have been proposed to exist in *Arabidopsis* (Kim and Delaney, 2002; also see discussion about these pathways in Liu et al., 2005) and probably in *B. napus* as well. Although the effect of two different SAR pre-treatments were not similar in terms of the effectiveness of resistance, the fact that both the treatments conferred resistance to *Psm* and *L. maculans* suggests that they may activate the same signaling pathways in *B. napus*. *B. napus* plants are particularly prone to *L. maculans* infection at the seedling stage and my results demonstrate that SAR is effective at this stage. I showed that enhanced resistance in seedlings is associated with *PR* gene induction and requires SA. Similar to what was observed in older plants, BTH was very effective in reducing disease caused by *L. maculans* at the seedling stage.

Overexpression of SAR genes (*AtDIR1* and *BnDIR1*, Chapter 3; *AtNPR1* and *BnNPR1*, Chapter 4) led to enhanced disease resistance even without SAR pre-treatments. In Chapter 2, I showed that SAR is effective against *Psm* and *L. maculans*. Results from Chapter 3 indicate that overexpression of *AtDIR1* gene in *B. napus* led to enhanced disease resistance to *Psm* but not to *L. maculans*. These results suggest that different defense pathways may be activated by SAR treatments and transgenic overexpression of the aforementioned SAR regulators. Based on my results, chemical SAR induced by BTH appears to confer resistance against a broader host-range of pathogens (a bacterium and a fungus) than was achieved by over expression of key SAR genes (bacterial resistance only). A recent study also found that expression of *AtNPR1* in tomato was not effective against all pathogens tested: These plants were resistant to certain bacteria and fungi but were unable to display resistance against any of the viruses

tested (Lin et al., 2004). However, Lin et al. (2004) did not compare the effectiveness of *NPR1* expression against that of BTH.

Plants have a complex network of signal transduction pathways for defense against pathogen attack (Feys and Parker, 2000; Kunkel and Brooks, 2002; McDowell and Dangl, 2000; Thomma et al., 2001). *P. syringae* is a biotroph and it has been suggested that effective defense against biotrophic pathogens is mainly due to PCD in the host and the associated activation of defense responses that are regulated by SA-dependent signaling pathways (Glazebrook, 2005). In contrast, necrotrophic pathogens benefit from host cell death, so they are not limited by PCD and SA-dependent defenses, but rather by a different set of defenses activated by JA and ET signaling pathways. For example, SAR does not protect *Arabidopsis* against necrotrophic pathogens such as *A. brassicicola* and *B. cinerea* (Thomma et al., 1998). *L. maculans* is a hemi-biotroph, a parasite that requires living host cells during only part of its life cycle (Howlett et al., 2001). It is possible that resistance to this type of pathogen requires a different set of signaling pathways that are not activated adequately by expression of *AtDIR1* by itself but would be by BTH or *P. syringae* pre-treatment. Also, it has been shown that a combination of SA and JA dependent pathways are necessary to develop resistance against pathogens (Ton et al., 2002). I have not tested whether combining SA and JA pretreatments may be more effective at enhancing resistance to *L. maculans*.

Over expression of the SAR regulator *AtNPR1* has been shown to be a very promising approach for enhancing disease resistance in crop plants such as rice and tomato (Chern et al., 2001; Lin et al., 2004). The over expression of *AtDIR1*, *AtNPR1*, *BnDIR1* and *BnNPR1* in *B. napus* did not result in any kind of developmental abnormalities. However, the *B. napus* transgenic plants expressing *AtDIR1* may display constitutive *BnPR* gene expression which requires energy. Defense reactions operating in incompatible host-parasite interactions require large amounts of energy and may have a detrimental effect on plant growth and production (Gurr and Rushton, 2005). It may be more beneficial to conserve energy of the plants and future studies should consider the introduction of foreign genes under the control of pathogen inducible promoters of *PR* genes, *isoflavone reductase* or *PAL* so that the plants will make appropriate and necessary proteins only when required.

Transgenic *B. napus* plants over expressing *DIR1* genes displayed additional disease resistance after chemical SAR pre-treatments. This was most apparent in plants expressing *AtDIR1*. I did not test the combined effects of *NPR1* over expression and SAR pre-treatments. However, over expression of *AtNPR1* in *Arabidopsis* was reported to enhance the responsiveness of plants to BTH (Friedrich et al., 2001). One possible molecular mechanism to explain these phenomena is known as "priming". The "priming" hypothesis proposes that SAR-derived signals prime or condition the plant tissue to react with a faster and more intense induction of defense reactions after an infection. Shirasu et al. (1997) showed that physiological concentrations of SA (10-100 μM) had negligible effects when administered to soybean cell suspensions in the absence of a pathogen. However, the same concentrations of SA markedly enhanced the induction of defense gene transcripts and hypersensitive cell death when the cultures were inoculated with avirulent *P. syringae* pv. *glycinea*. Support for a role of SA in priming was first demonstrated in elicitor-induced parsley cell cultures (Conrath et al., 2002). *Arabidopsis* plants pre-treated with avirulent pathogens or BTH showed an increase in sensitivity to *P. syringae*-induced activation of *PAL* and callose deposition, two processes that are not induced by BTH alone (Kohler et al., 2002). In transgenic *B. napus* plants, SA that is produced at the site of infection during biological SAR, or the presence of the SA functional analog BTH during chemical SAR, might be producing a similar effect and leading to the additional increase in resistance against *Psm*. Higher levels of SAR genes present in the transgenic plants, or the introduction of key SAR genes from heterologous plants having slightly different functions or regulatory properties, in combination with the SAR pre-treatments could be contributing to increase the effectiveness of SAR responses. This also suggests that even after SAR activation by pre-treatments, in particular spraying with BTH, the activity of the endogenous DIR1 and NPR1 proteins continue to be limiting factors affecting the effectiveness of SAR in untransformed plants.

Additional experimentation will be required to determine whether the transgenic *B. napus* plants that I generated are more responsive to BTH, and the minimal effective level of this activator that can be used in conjunction with these transgenic plants. The *Arabidopsis* plants over expressing *AtNPR1* were also reported to display enhanced efficiency to reduced use of three different fungicides that were tested (Friedrich et al.,

2001). Since pathogens are exposed to lower amounts of fungicide, it would reduce the chances of the pathogen becoming resistant to these fungicides. It will be interesting to test whether *B. napus* plants expressing DIR1 or NPR1 respond similarly. Using large amounts of chemical SAR activator, pesticides and fungicides can be expensive. Furthermore, pesticides and fungicide use is taxing on the environment. Therefore, use of disease resistant plants in combination with reduced amounts of chemical inducers of SAR such as BTH has several potential benefits in terms of plant disease management.

Sequence similarity of *BnNPR1* to *AtNPR1* and functional complementation of the disease resistance phenotypes in the *Arabidopsis npr1* mutant plants by *BnNPR1* (Chapter 4) indicate that similar to *AtNPR1*, *BnNPR1* may be involved in signaling during SAR in *B. napus*. Understanding plant signal transduction pathways during SAR (see Figure 1.1) is nowhere near complete. To get more insight into the molecular mechanisms of *BnNPR1* and *BnDIR1* genes, particularly in the context of SAR, it will be important to study their regulatory mechanisms and also characterize the biochemical structure and function of the encoded proteins.

Whereas wild-type *Arabidopsis* plants express *PR* genes in systemic leaves following inoculation with avirulent *Pst*, the *dir1-1* mutants do not (Maldonado et al., 2002). Furthermore, phloem exudates from infected wild-type plants, but not *dir1-1* plants, induce *PR* gene expression when infiltrated into leaves of wild-type plants (Maldonado et al., 2002). It will be very important to confirm whether systemic expression of *PR* genes is restored in the *Arabidopsis dir1-1* mutant plants expressing *BnDIR1*, and whether exudates from these plants can induce *PR* gene expression upon infiltration into wild-type leaves. This information will be critical in determining whether *BnDIR1* can indeed complement the *dir1-1* mutation. Also, it will be interesting to see if the phloem exudates from uninfected transgenic plants (*Arabidopsis* or *B. napus*) expressing *DIR1* can activate defense responses since these plants display higher levels of disease resistance even without SAR treatments.

Over expression is one of the strategies to study the function of a gene. In my thesis, I have overexpressed four different SAR genes (*AtDIR1* and *BnDIR1*, Chapter 3; *AtNPR1* and *BnNPR1*, Chapter 4) and the resulting plants displayed enhanced disease resistance against *P. syringae*. However, all the over expressing lines did not behave

similarly and it was difficult at times to interpret results. Therefore, it may be beneficial to study the functions of a gene using several other methods. In addition to over expression, gene suppression (deletion/mutation) (Matzke and Matzke, 1995) and gene disruption are powerful and direct tools for obtaining loss-of-function mutants that help in ascertaining biological function of the numerous uncharacterized genes. TILLING (Targeting Induced Local Lesions In Genomes) is one of the reverse genetic strategies that involves chemical mutagenesis followed by screening for point mutations (Till et al. 2003; Henikoff et al. 2004). Unlike T-DNA mutagenesis, TILLING does not require the generation of large numbers (>100,000 individuals) of independent transgenic plants. It also allows the recovery of multiple point mutations in any given gene, leading to the generation of allelic series that can help to better ascertain the function of a gene. Because of the large genome size in *B. napus*, and the presence of large families of highly-related genes (typically, for every gene found in the *Arabidopsis* genome, there is potentially 6 gene equivalent in *B. napus*), functional redundancy can be a serious limitation of TILLING. Mutations in multiple related genes must first be identified, and probably combined before a phenotype becomes apparent. RNA interference (RNAi) can facilitate the simultaneous silencing of multiple related genes (Chandler and Werr, 2003) and could be useful for studying genes in *B. napus*. Attempts were made to make construct RNAi vectors for silencing *BnDIR1* and *BnNPR1*. I had difficulties cloning the chosen fragments of both the genes in diverging orientation into the pKANNIBAL vector. This was likely due to the close proximity of restriction sites available on the pKANNIBAL vector and to the instability of inverted repeats in *E. coli*. Use of GATEWAY-compatible RNAi vectors would facilitate the cloning of inserts; however, none were readily accessible at the time.

Transformation of *B. napus* is a time consuming process and takes at least 6-8 months. Therefore, virus-induced gene silencing (VIGS) that involves the suppression of targeted gene transcripts and that does not require the production of stable transgenic plants (Burch-Smith et al., 2004) might be able to offer a different alternative to characterize the function of genes in *B. napus*. Using VIGS, Liu et al. (2002a) demonstrated in tobacco the necessity of several genes including *NtNPR1* for *R*-gene-mediated resistance against Tobacco Mosaic Virus (specifically the *R*-gene, *N*).

Similarly, Ekengren et al. (2003) used VIGS to show that several other genes, including *LeNPR1*, are necessary for *R*-gene-mediated resistance in tomato (specifically, *Pto*). Therefore, it may be quite feasible to use VIGS for studying the functions of *BnDIR1* and *BnNPR1* in *B. napus* plants.

Genes are regulated temporally and/or spatially in response to various external and internal stimuli. The expression of each gene results from specific *cis*-regulatory elements, especially in the 5' non-coding region containing the promoter. At this moment nothing is known as to how the *BnDIR1* and *BnNPR1* genes are regulated. Therefore, characterization of the *BnDIR1* and *BnNPR1* promoters may be able to determine which *cis*-elements are important for promoter function and allow transcriptional regulation of these genes. The full-length and deleted versions of the promoters can be tagged to green fluorescent protein (GPF) and promoter activity of all the constructs can be assayed under control (no SAR) and SAR inducing (biological and chemical) conditions.

Using micro array analysis, changes in gene expression in *Arabidopsis* plants under several different SAR-inducing or SAR-repressing conditions were monitored (Maleck et al., 2000). There were 213 genes that were differentially regulated under these conditions. *B. napus* micro arrays representing 13,000 ESTs are available with Biotechnology and Biological Sciences Research Council (BBSRC), UK and AAFC, Saskatoon (http://www.brassica.info/meetings/mbgp_sc_jan_2005.doc). Micro array experiments using systemic leaf tissues of *B. napus* after various times of infection with avirulent *Psm* or BTH treatment would give an idea as to how SAR and SAR-related genes are regulated in the plants before and after SAR induction. Comparing plants that are treated with BTH with those over expressing various transgenes that I used in my study could help resolve whether BTH and *DIR1*/*NPR1* induce different pathways. I have observed that *B. napus* plants over expressing *AtDIR1* may be able to express *BnPR* genes constitutively. Comparison of *B. napus* plants overexpressing different SAR genes with wild-type plants with and without biological or chemical induction of SAR may help determine, and provide stronger evidence as to, whether the defense pathways are activated constitutively. These results can also be used to confirm priming in *B. napus* plants overexpressing various SAR genes.

An enhanced understanding of protein-protein interactions is important to the successful elucidation of multiprotein pathways, which mediate a vital process such as SAR. Studies in *Arabidopsis* have indicated the presence of several AtNPR1 interacting proteins called NIMINs, which appear to be negative regulators of SAR (Weigel et al., 2005). It is quite possible that *B. napus* may contain orthologs of these NIMIN proteins that interact with BnNPR1. Also, there is no report on any interactors of AtDIR1 so far. Therefore, the yeast two-hybrid system can be used to identify proteins that interact with BnNPR1 and BnDIR1 (Chien et al., 1991). In planta interactions can also be assayed by immuno-precipitation of BnDIR1 and BnNPR1 associated proteins from plant extracts using antibodies raised against peptides of BnDIR1 and BnNPR1 (Buck and Lieb, 2004).

Biochemical characterization of proteins is another powerful tool to study the functioning of proteins. AtDIR1 is known to be involved in early signaling events and AtNPR1 is known to function in the later part of the signal transduction pathway during SAR. The putative protein structure of DIR1 indicates that it is a LTP. Therefore, it has been suggested that it may be involved in transferring the signaling molecule from the pathogen to the plant (Maldonado et al., 2002). It will be interesting to see where in the cell this process may be taking place and also investigate how and when BnDIR1 is carrying a signal in the plant. Nuclear localization of NPR1 is linked to the reduction of two of its ten conserved cysteines (C82 and C216) and requires the NLS (Kinkema et al., 2000; Mou et al., 2003). Both the cysteines corresponding to C82 and C216 as well as the NLS are present in the predicted BnNPR1 protein. It will therefore be interesting to see if the BnNPR1 localizes in a similar fashion. To accomplish these objectives, an in-frame fusion between the full-length *BnDIR1* and *BnNPR1* genes with GFP can be created under the control of their own promoters and transformed into their respective *Arabidopsis* mutants. After different SAR induction treatments, observations can be made using high resolution confocal microscopy through a time-course after SAR treatments to follow the changes in cellular localization patterns of these two proteins. Particularly, in the case of BnDIR1, it will be interesting to look at the whole plant with a special focus on the veins and stems of the plants. If the *BnDIR1* gene is involved very early in defense signaling, the BnDIR1 protein would be localized on the cell membranes and/or

cytoplasm of the cells. Also, if the signal is carried through the phloem, it should be possible to track the direction and movement of the protein through the vascular tissue.

Northern analysis on all the *B. napus* plants overexpressing SAR genes indicated that there was no correlation between transgene expression and the level of disease resistance achieved. Indirectly, the expression of genes of interest can be measured using micro arrays that can provide a rough measure of the cellular concentration of different mRNAs, with real-time PCR being a more sensitive and accurate method for measuring relative expression of specific genes. However, the expression of many genes is known to be regulated after transcription, so mRNA concentration may not necessarily be an accurate measure of the amount of active protein. Thus, it is possible that better correlation between disease resistance and transgene expression will be revealed by monitoring protein levels by western blot analysis. Also, it might be interesting to measure the complete metabolic profile of the transgenic plants and compare it to the profile of the non-modified plant, so that changes due to the genetic modification can be analyzed to make sure of any unexpected beneficial changes such as increase in levels of nutrients or vitamins or changes in unnecessary compounds.

Use of molecular and genome technology in plant-microbe interactions, particularly in the model plants, has generated a vast expanse of information and aided in the better understanding of genetics, biochemistry and physiology of disease resistance mechanisms. However, similar research on crop plants such as *B. napus*, especially at the molecular level, has lagged behind. Therefore, a lot more research needs to be done to clearly understand the underlying signal transduction mechanisms involved in establishing disease resistance in *B. napus*.

CHAPTER 6. Conclusions

There is a wealth of information on the molecular biology of SAR in *Arabidopsis*. With this as a guide, I set up the major goal of my thesis to characterize the molecular aspects of this phenomenon in the crop plant *B. napus*. The following are the major conclusions that I was able to draw from my work.

1. Induction of SAR in *B. napus* is effective at enhancing resistance against virulent strains of the bacterial pathogen *Psm* and the fungal pathogen *L. maculans*. Chemical SAR, induced by BTH, was much more effective than biological SAR triggered by localized pre-inoculation with an avirulent pathogen. However, both treatments resulted in long lasting (3 week) resistance.

2. SAR in *B. napus* displays the hallmarks of the classical phenomenon reported in *Arabidopsis* and other model plants. In addition to being long lasting and broad range (see #1), it is correlated with the expression of *PR* genes, including *BnPR-1* and *BnPR-2*. Studies with *NahG* plants demonstrated the requirement for SA; however, considerable residual *PR* gene expression was detected in the *NahG* plants, suggesting a role for SA-independent signaling in the regulation of these genes.

3. The *B. napus* genome contains genes that are highly related to the key SAR regulators *AtDIR1* and *AtNPR1*. Based on its ability to functionally complement *Arabidopsis* mutants, it was concluded that the *Brassica NPR1*-related gene isolated in this study (*BnNPR1*) is orthologous to *AtNPR1*. Evidence that the *Brassica DIR1*-related gene (*BnDIR1*) complements the *Arabidopsis dir1-1* mutant could not be obtained. However, expression of *BnDIR1* in *dir1-1* and wild-type *Arabidopsis* plants enhanced basal resistance against *Pst*, complicating the analysis of SAR. The potential role of *DIR1* in basal resistance is an important and novel finding which needs to be further characterized.

4. Overexpression of SAR genes (*AtDIR1*, *BnDIR1*, *AtNPR1* and *BnNPR1*) in transgenic *B. napus* was found to be effective at enhanced disease resistance against *Psm* even without SAR pre-treatments (i.e. basal resistance), but generally ineffective at increasing disease resistance against the fungal pathogen *L. maculans*. Given that BTH treatment was highly effective against *L. maculans* (see #1), it was concluded that the defense responses triggered by this chemical could differ from those regulated by overexpression of *DIR1* and *NPR1* genes.

5. Enhanced resistance observed in transgenic *B. napus* plants expressing *AtDIR1* may be associated with the constitutive expression of *BnPR* genes, and accordingly, due to constitutive activation of defense responses. These plants displayed additional disease resistance after treatment with BTH. Therefore, the combination of these two manipulations may result in more effective protection against pathogens under field conditions and provide a possibility for better disease management.

CHAPTER 7. References

Aarts, N., Metz, M., Holub, E., Staskawicz, B.J., Daniels, M.J., and Parker, J.E. (1998) Different requirements for EDS1 and NDR1 by disease resistance genes define at least two *R* gene-mediated signaling pathways in *Arabidopsis*. Proc. Natl. Acad. Sci. U S A. **95**: 10306-10311.

Ackman, R.G. (1990) Canola fatty acids: an ideal mixture for health, nutrition, and food use. In: F. Shahidi (Ed.) Canola and rapeseed, production, chemistry, nutrition and processing technology. Van Nostrand Reinhold, New York, USA. pp. 81-98.

Agrios, G.N. (1997) Plant Pathology, 4th Ed., Academic Press Inc., San Diego, USA. 635 pp.

Alexander, D., Goodman, R.M., Gut-Rella, M., Glascock, C., Weymann, K., Friedrich, L., Maddox, D., Ahl-Goy, P., Luntz, T., Ward, E., and Ryals, J. (1993) Increased tolerance to two oomycete pathogens in transgenic tobacco expressing pathogenesis-related protein 1a. Proc. Natl. Acad. Sci. USA. **90**: 7327-7331.

Alfano, J.R., and Collmer, A. (2004) Type III secretion system effector proteins: Double agents in bacterial disease and plant defense. Annu. Rev. Phytopathol. **42**: 385-414.

Alfano, J.R., and Collmer, A. (1997) The type III (Hrp) secretion pathway of plant pathogenic bacteria: trafficking harpins, Avr proteins, and death. J. Bacteriology **179**: 5655-5662.

Alvarez, M.E., Pennell, R.I., Meijer, P.J., Ishikawa, A., Dixon, R.A., and Lamb, C. (1998) Reactive oxygen intermediates mediate a systemic signal network in the establishment of plant immunity. Cell **92**: 773-784.

Antoniw, J., and White, R. (1980) The effects of aspirin and polyacrylic acid on soluble leaf proteins and resistance to virus infection in five cultivars of tobacco. Phytopathol. Z. **98**: 331-341.

***Arabidopsis* Genome Initiative** (2000) Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. Nature **408**: 796-815.

Aravind, L., and Koonin, E.V. (1999) Fold prediction and evolutionary analysis of the POZ domain: Structural and evolutionary relationship with the potassium channel tetramerization domain. J. Mol. Biol. **285**: 1353-1361.

Ausubel, F.M. (2005) Are innate immune signaling pathways in plants and animals conserved? Nature Immunol. **10**: 973-979.

Banerjee, D., Zhang, X., and Bent, A.F. (2001) The leucine-rich repeat domain can determine effective interaction between RPS2 and other host factors in *Arabidopsis* RPS2-mediated disease resistance. Genetics **158**: 439-450.

Beckers, G.J., and Spoel, S.H. (2006) Fine-tuning plant defence signalling: salicylate versus jasmonate. Plant Biol. **8**: 1-10.

Beisson, F., Koo, A.J.K., Ruuska, S., Schwender, J., Pollard, M., Thelen, J.J., Paddock, T., Salas, J.J., Savage, L., Milcamps, A., Mhaske, V.B., Cho, Y., and Ohlrogge, J.B. (2003) A 2003 census of the candidates, a study of the distribution of expressed sequence tags in organs, and a web-based data base. Plant Physiol. **132**: 681-697.

Bonas, U., and Lahaye, T. (2002) Plant disease resistance triggered by pathogen-derived molecules: refined models of specific recognition. Curr. Opin. Microbiol. **5**: 44-50.

Borges, A.A., Cools, H.J., and Lucas, J.A. (2003) Menadione sodium bisulphate: a novel plant defence activator which enhances local and systemic resistance to infection by *Leptosphaeria maculans* in oil seed rape. Plant Pathol. **52**: 429-436.

Bowling, S.A., Clarke, J.D., Liu, Y., Klessig, D.F., and Dong, X. (1997) The *cpr5* mutant of *Arabidopsis* expresses both NPR1-dependent and NPR1-independent resistance. Plant Cell **9**: 1573-1584.

Bowling, S.A., Guo, A., Cao, H., Gordon, A.S., Klessig, D.F., and Dong, X. (1994) A mutation in *Arabidopsis* that leads to constitutive expression of systemic acquired resistance. Plant Cell **6:** 1845-1857.

Broekaert, W.F., Terras, F.R.G., Cammue, B.P.A., and Osborn, R.W. (1995) Plant defensins: Novel antimicrobial peptides as components of the hoist defense system. Plant Phsyiol. **108**: 1353-1358.

Broglie, K., Chet, I., Holliday, M., Cressman, R., Biddle, P., Knowlton, S., Mauvais, C.J., and Broglie, R. (1991) Transgenic plants with enhanced resistance to the fungal pathogen *Rhizoctonia solani*. Science **254**: 1194-1197.

Buck, M.J., and Lieb, J.D. (2004) ChIP-chip: considerations for the design, analysis, and application of genome-wide chromatin immunoprecipitation experiments. Genomics **83**: 349-360.

Burch-Smith, T.M., Anderson, J.C., Martin, G.B., and Dinesh-Kumar, S.P. (2004) Applications and advantages of virus-induced gene silencing for gene function studies in plants. Plant J. **39**: 734-746.

Cameron, R.K. (2000) Salicylic acid and its role in plant defense responses: What do we really know? Physiol. Mol. Plant Pathol. **56**: 91-93.

Cameron, R.K., Dixon, R.A., and Lamb, C. (1994) Biologically induced systemic acquired resistance in *Arabidopsis thaliana.* Plant J. **5**: 715-725.

Cameron, R.K., Paiva, N.L., Lamb, C.J., and Dixon, R.A. (1999) Accumulation of salicylic acid and *PR-1* gene transcripts in relation to the systemic acquired resistance (SAR) response induced by *Pseudomonas syringae* pv. *tomato* in *Arabidopsis*. Physiol. Mol. Plant Pathol. **55**: 121-130.

Campbell, M.A., Fitzgerald, H.A., and Ronald, P.C. (2002) Engineering pathogen resistance in crop plants. Transgenic Res. **11**: 599-613.

Castro, M.S., and Fontes, W. (2005) Plant defense and antimicrobial peptides. Protein Peptide Letters **12**: 11-16.

Cao, H., Bowling, S.A., Gordon, A.S., and Dong, X. (1994) Characterizaton of an *Arabidopsis* mutant that is nonresponsive to inducers of systemic acquired resistance. Plant Cell **6**: 1583-1592.

Cao, H., Glazebrook, J., Clarke, J.D., Volko, S., and Dong, X. (1997) The *Arabidopsis* NPR1 gene that controls systemic acquired resistance encodes a novel protein containing ankyrin repeats. Cell **88**: 57-63.

Cao, H., Li, X., and Dong, X. (1998) Generation of broad-spectrum disease resistance by overexpression of an essential regulatory gene in systemic acquired resistance. Proc. Natl. Acad. Sci. USA. **95**: 6531-6536.

Cavell, A.C., Lydiate, D.J., Parkin, I.A.P., Dean, C., and Trick, M. (1998) Collinearity between a 30-centimorgan segment of *Arabidopsis thaliana* chromosome 4 and duplicated regions within the *Brassica napus* genome. Genome **41**: 62-69.

Chandler, J.W., and Werr, W. (2003) When negative is positive in functional genomics. Trends Plant Sci. **8**: 279-285.

Cheong, N.E., Choi, Y.O., Kim, W.Y., Kim, S.C., Cho, M.J., and Lee, S.Y. (1997) Purification of an antifungal PR-5 protein from flower buds of *Brassica campestris* and cloning of its gene. Physiol. Plantarum **101**: 583-590.

Chern, M., Canlas, P.E., Fitzgerald, H.A., and Ronald, P.C. (2005a) Rice NRR, a negative regulator of disease resistance, interacts with *Arabidopsis* NPR1 and rice NH1. Plant J. **43**: 623-635.

Chern, M., Fitzgerald, H.A., Canlas, P.E., Navarre, D.A., and Ronald, P.C. (2005b) Overexpression of a rice NPR1 homolog leads to constitutive activation of defense response and hypersensitivity to light. Mol. Plant-Microbe Interact. **18**: 511-520.

Chern, M.S., Fitzgerald, H.A., Yadav, R.C., Canlas, P.E., Dong, X., and Ronald, P.C. (2001) Evidence for a disease-resistance pathway in rice similar to the NPR1- mediated signaling pathway in *Arabidopsis*. Plant J. **27**: 101-113.

Chien, C.T., Bartel, P.L., Sternglanz, R., and Fields, S. (1991) The two-hybrid system: a method to identify and clone genes for proteins that interact with a protein of interest. Proc. Natl. Acad. Sci. USA. **88**: 9578-9582.

Clarke, J.D., Liu, Y., Klessig, D.F., and Dong, X. (1998) Uncoupling *PR* gene expression from *NPR1* and bacterial resistance: Characterization of the dominant *Arabidopsis cpr6-1* mutant. Plant Cell **10**: 557-569.

Clough, S.J., and Bent, A.F. (1998) Floral dip: a simplified method for *Agrobacterium* mediated transformation of *Arabidopsis thaliana*. Plant J. **16**: 735-743.

Cohen, Y., Peer, S., Balass, O., and Coffey, M. (1987) A fluorescent technique for studying growth of *Peronospora tabacina* on leaf surfaces. Phytopathol. **77:** 201-204.

Cohen, Y. (2002) *β*-aminobutyric acid-induced resistance against plant pathogens. Plant Disease **86**: 448-457.

Cohen, Y., and Gisi, U. (1994) Systemic translocation of 14C-DL-3-aminobutyric acid in tomato plants in relation to induced resistance against *Phytophthora infestans*. Physiol. Mol. Plant Pathol. **45**: 441-456.

Collmer A., Badel, J.L., Charkowski, A.O., Deng, W.L., Fouts, D.E., Ramos, A.R., Rehm, A.H., Anderson, D.M., Schneewind, O., van Dijk, K., and Alfano, J.R. (2000) *Pseudomonas syringae* Hrp type III secretion system and effector proteins. Proc. Natl. Acad. Sci. USA. **97:** 8770-8777.

Conrath, U., Thulke, O., Katz, V., Schwindling, S., and Kohler, A. (2001) Priming as a mechanism in induced systemic resistance of plants. Eur. J. Plant Pathol. **107:** 113-119.

Conrath, U., Pieterse, C.M., and Mauch-Mani, B. (2002) Priming in plant-pathogen interactions. Trends Plant Sci. **7**: 210-216.

Constantinescu, O., and Fatehi, J. (2002) *Peronospora*-like fungi (Chromista, Peronosporales) parasitic on Brassicaceae and related hosts. Nova Hedwigia **74**: 291-338.

Coppinger, P., Repetti, P.P., Day, B., Dahlbeck, D., Mehlert, A., and Staskawicz, B.J. (2004) Overexpression of the plasma membrane-localized NDR1 protein results in enhanced bacterial disease resistance in *Arabidopsis thaliana*. Plant J. **40**: 225-237.

Crute, I.R. (1986) The genetic basis of relationships between microbial parasites and their hosts. In Mechanisms of resistance in plant diseases (ed. R.S.S. Fraser), Martinus Nijhoff and W. Junk, Dordrecht, The Netherlands. pp. 80-142.

Dangl, J.L., Dietrich, R.A., and Richberg, M.H. (1996) Death don't have no mercy: Cell Death Programs in Plant-Microbe Interactions. Plant Cell **8**: 1793-1807.

Dangl, J.L., and Jones, J.D. (2001) Plant pathogens and integrated defence responses to infection. Nature **411**: 826-833.

Dann, E.K., and Deverall, B.J. (1995) Effectiveness of systemic resistance in bean against foliar and soil-borne pathogens as induced by biological and chemical means. Plant Pathol. **44**: 458-466.

Delaney, T.P., Uknes, S., Vernooij, B., Friedrich, L., Weymann, K., Negrotto, D., Gaffney, T., Gur-Rella, M., Kessmann, H., Ward, E., and Ryals, J. (1994) A central role of salicylic acid in plant disease resistance. Science **266**: 1247-1250.

Delaney, T.P., Friedrich, L., and Ryals, J.A. (1995) *Arabidopsis* signal transduction mutant defective in chemically and biologically induced disease resistance. Proc. Natl. Acad. Sci. USA. **92**: 6602-6606.

Dempsey, D.A., Shah, J., and Klessig, D.F. (1999) Salicylic acid and disease resistance in plants. Crit. Rev. Plant Sci. **18**: 547-575.

Després, C., Chubak, C., Rochon, A., Clark, R., Bethune, T., Desveaux, D., and Fobert, P.R. (2003) The *Arabidopsis* NPR1 disease resistance protein is a novel cofactor that confers redox regulation of DNA binding activity to the basic domain/leucine zipper transcription factor TGA1. Plant Cell **15**: 2181-2191.

Després, C., DeLong, C., Glaze, S., Liu, E., and Fobert, P.R. (2000) The *Arabidopsis* NPR1/NIM1 protein enhances the DNA binding activity of a subgroup of the TGA family of bZIP transcription factors. Plant Cell **12**: 279-290.

Dewdney, J., Reuber, T.L., Wildermuth, M.C., Devoto, A., Cui, J., Stutius, L.M., Drummond, E.P., and Ausubel, F.M. (2000) Three unique mutants of *Arabidopsis* identify *eds* loci required for limiting growth of a biotrophic fungal pathogen. Plant J. **24**: 205-218.

Dong, X. (2001) Genetic dissection of systemic acquired resistance. Curr. Opin. Plant Biol. **4**: 309-314.

Dong, X. (2004) NPR1, all things considered. Curr. Opin. Plant Biol. **7**: 547-552.

Dong, X., Li, X., Zhang, Y., Fan, W., Kinkema, M., and Clarke, J. (2001) Regulation of systemic acquired resistance by NPR1 and its partners. Novartis Found Symp. **236**: 165-173.

Douliez, J.P., Michon, T., Elmorjani, K., and Marion, D. (2000) Structure, biological and technological functions of lipid transfer proteins and indolines, the major lipid binding proteins from cereal kernels. J. Cereal Sci. **31**: 1-20.

Du, H., and Klessig, D.F (1997) Identification of a soluble, high-affinity salicylic acid-binding protein in tobacco. Plant Physiol. **113**: 1319-1327.

Durrant, W.E., and Dong, X. (2004) Systemic acquired resistance. Annu. Rev. Phytopathol. **42**: 185-209.

Eckardt, N.A., and Innes, R. (2003) Resistance rodeo: Rounding up the full complement of *Arabidopsis NBS-LRR* genes. Plant Cell **15**: 806-807.

Economic Research Service (ERS) (2001) Oil crops situation and outlook. OCS-2000, Oct. 2001. ERS, USDA. pp. 66.

Edqvist, J., and Farbos, I. (2002) Characterization of germination-specific lipid transfer proteins from *Euphorbia lagascae*. Planta **215**: 41-50.

Ekengren, S.K., Liu, Y., Schiff, M., Dinesh-Kumar, S.P., and Martin, G.B. (2003) Two MAPK cascades, NPR1, and TGA transcription factors play a role in Pto-mediated disease resistance in tomato. Plant J. **36**: 905-917.

Enyedi, A.J., Yalpani, N., Silverman, P., and Raskin, I. (1992) Signal molecules in systemic plant resistance to pathogens and pests. Cell **70**: 879-886.

Eulgem, T. (2005) Regulation of the *Arabidopsis* defense transcriptome. Trends Plant Sci. **10**: 71-78.

Falk, A., Feys, B.J., Frost, L.N., Jones, D.G., Daniels, M.J., and Parker, J. (1999) EDS1, an essential component of *R*-gene mediated disease resistance in *Arabidopsis*, has homology to eukaryotic lipases. Proc. Natl. Acad. Sci. USA. **96:** 3292-3297.

Fan, W., and Dong, X. (2002) In vivo interaction between NPR1 and transcription factor TGA2 leads to salicylic acid-mediated gene activation in *Arabidopsis*. Plant Cell **14**: 1377-1389.

FAO, FAOSTAT (2004) FAO statistical databases. Rome, Italy: FAO, UN. http: //faostat.fao.org

Felix, G., Duran, J.D., Volko, S., and Boller, T. (1999) Plants have a sensitive perception system for the most conserved domain of bacterial flagellin. Plant J. **18**: 265-276.

Ferrari, S., Plotnikova, J.M., De Lorenzo, G., and Ausubel, F.M. (2003) *Arabidopsis* local resistance to *Botrytis cinerea* involves salicylic acid and camalexin and requires EDS4 and PAD2, but not SID2, EDS5 or PAD4. Plant J. **35**: 193-205.

Feys, B.J., Moisan, L.J., Newman, M.A., and Parker, J.E. (2001) Direct interaction between the *Arabidopsis* disease resistance signaling proteins, EDS1 and PAD4. EMBO J. **20**: 5400-5411.

Feys, B.J., Wiermer, M., Bhat, R.A., Moisan, L.J., Medina-Escobar, N., Neu, C., Cabral, A., and Parker, J.E. (2005) *Arabidopsis* SENESCENCE-ASSOCIATED GENE101 stabilizes and signals within an ENHANCED DISEASE SUSCEPTIBILITY1 complex in plant innate immunity. Plant Cell **17**: 2601-2613.

Feys, B.J., and Parker, J.E. (2000) Interplay of signaling pathways in plant disease resistance. Trends Genet. **16**: 449-455.

Fitzgerald, H.A., Chern, M.S., Navarre, R., and Ronald, P.C. (2004) Overexpression of *(At)NPR1* in rice leads to a BTH- and environment-induced lesion-mimic/cell death phenotype. Mol. Plant-Microbe Interact. **17**: 140-151.

Flor, H.H. (1942) Inheritance of pathogenicity in *Melampsora lini*. Phytopathol. **32**: 653-669.

Fobert, P.R., and Després, C. (2005) Redox control of systemic acquired resistance. Curr. Opin. Plant Biol. **8**: 378-382.

Forouhar, H., Yang, Y., Kumar, D., Chen, Y., Fridman, E., Park, S.W., Chiang, Y., Acton, T.B., Montelione, G.T., Pichersky, E., Klessig, D.F., and Tong, L. (2005) Structural and biochemical studies identify tobacco SABP2 as a methyl salicylate esterase and implicate it in plant innate immunity. Proc. Natl. Acad. Sci. USA. **102**: 1773-1778.

Foster, E., Hattori, J., Labbe, H., Ouellet, T., Fobert, P., James, L., and Miki, B. (1999) A tobacco cryptic constitutive promoter, *tCUP*, revealed by T-DNA tagging. Plant Mol. Biol. **41:** 45-55.

Friedrich, L., Lawton, K., Ruess, W., Masner, P., Specker, N., Gut-Rella, M., Meier, B., Dincher, S., Staub, T. and Uknes, S., Métraux, J.P., Kessmann, H., and Ryals, J. (1996) A benzothiadiazole derivative induces systemic acquired resistance in tobacco. Plant J. **10**: 61-70.

Friedrich, L., Lawton, K., Dietrich, R., Willits, M., Cade, R., and Ryals, J. (2001) *NIM1* overexpression in *Arabidopsis* potentiates plant disease resistance and results in enhanced effectiveness of fungicides. Mol. Plant-Microbe Interact. **14**: 1114-1124.

Fristensky, B., Balcerzak, M., Daifen, H., and Zhang, P. (1999) Expressed sequence tags from the defense response of *Brassica napus* L. to *Leptosphaeria maculans*. Mol. Plant Pathol. On-line http: //www.bspp.org.uk/mppol/1999/0301fristensky.

Fromm, H., Katagiri, F., and Chua, N-H. (1989) An octopine synthase enhancer element directs tissue-specific expression and binds ASF-1, a factor from tobacco nuclear extracts. Plant Cell **1**: 977-984.

Gaffney, T.P., Friedrich, L., Vernooij, B., Negrotto, D., Neye, G., Uknes, S., Ward, E., Kessmann, H., and Ryals, J. (1993) Requirement of salicylic acid for the induction of systemic acquired resistance. Science **261**: 754-756.

Galan J.E., and Collmer, A. (1999) Type III secretion machines: bacterial devices for protein delivery into host cells. Science **284**: 1322-1328.

Garcia-Olmedo, F., Molina, A., Segura, A., and Moreno, M. (1995) The defensive role of nonspecific lipid-transfer proteins in plants Trends Microbiol. **3**: 72-74.

Gianinazzi, S., and Ahl, P. (1983) The genetic and molecular basis. of b-proteins in the genus *Nicotiana*. Neth. J. Plant. Pathol. **89**: 275-283.

Glazebrook, J. (2001) Genes controlling expression of defense responses in *Arabidopsis*-2001 status. Curr. Opin. Plant Biol. **4**: 301-308.

Glazebrook, J. (2005) Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. Annu. Rev. Phytopathol. **43**: 205-227.

Glazebrook, J., Rogers, E.E., and Ausubel, F.M. (1996) Isolation of *Arabidopsis* mutants with enhanced disease susceptibility by direct screening. Genetics **143**: 973-982.

Glazebrook, J., Zook, M., Mert, F., Kagan, I., Rogers, E.E., Crute, I.R. Holub, E.B., Hammerschmidt, R., and Ausubel, F.M. (1997) Phytoalexin-deficient mutants of *Arabidopsis* reveal that PAD4 encodes a regulatory factor and that four PAD genes contribute to downy mildew resistance. Genetics **146**: 381-392.

Godard, J.F., Ziadi, S., Monot, C., Le Corre, D., and Silue, D. (1999) Benzothiadiazole (BTH) induces resistance in cauliflower (*Brassica oleracea* var. *botrytis*) to downy mildew of crucifers caused by *Peronospora parasitica*. Crop Prot. **18**: 397-405.

Görlach, J., Volrath, S., Knauf-Beiter, G., Hengy, G., Beckhove, U., Kogel, K.H., Oostendorp, M., Staub, T., Ward, E., Kessman, H., and Ryals, J. (1996) Benzothiadiazole, a novel class of inducers of systemic acquired resistance, activates gene expression and disease resistance in wheat. Plant Cell **8**: 629-643.

Greenberg, J.T. (1997) Programmed cell death in plant-pathogen interactions. Annu. Rev. Plant Physiol. Plant Mol. Biol. **48**: 525-545.

Guedes, M.E.M., Richmond, S., and Kuć, J. (1980) Induced systemic resistance to anthracnose in cucumber as influenced by the location of the inducer inoculation with *Colletotrichum lagenarium* and the onset of flowering and fruiting. Physiol. Plant Pathol. **17:** 229-233.

Gurr, S.J., and Rushton, P.J. (2005) Engineering plants with increased disease resistance: what are we going to express? Trends Biotechnol. **23**: 275-282.

Hahlbrock, K., and Scheel, D. (1989) Physiology and molecular biology of phenylpropanoid metabolism. Annu. Rev. Plant Physiol. Plant Mol. Biol. **40**: 347-369.

Hammerschmidt, R. (1999). Induced disease resistance: how do induced plants stop pathogens? Physiol. Mol. Plant Pathol. **55**: 77-84.

Hammerschmidt, R., and Becker, J.S. (1997) Acquired resistance to disease in plants. Hortic. Rev. **18**: 247-288.

Hammerschmidt, R., and Kuć, J. (1995) Induced resistance to diseases in plants. Kluwer Academic Press, Dordrecht, The Netherlands.

Hammerschmidt, R., and Yang-Cashman, P. (1995) Induced resistance in cucurbits. In: R. Hammerschmidt & J. Kuć (eds), Induced Resistance to Disease in Plants. Kluwer, Boston, USA. pp. 63-85.

Heath, M.C. (2000) Non-host resistance and nonspecific plant defenses. Curr. Opin. Plant Biol. **3**: 315-319.

Heck, S., Grau, T., Buchala, A., Métraux, J.-P., and Nawrath, C. (2003) Genetic evidence that expression of *NahG* modifies defence pathways independent of salicylic acid biosynthesis in the *Arabidopsis-Pseudomonas syringae* pv. *tomato* interaction. Plant J. **36:** 342-352.

Hedge, I.C. (1976) A systematic and geographical survey of the world cruciferae. In: J.G. Vaughn, A.J. MacLeod, and B.M.G. Jones (eds.), The biology and chemistry of cruciferae. Academic Press, New York, USA. pp. 1-45.

Henikoff, S., Till, B.J., and Tilling, C.I. (2004) Traditional mutagenesis meets functional genomics. Plant Physiol. **135**: 630-636.

Hennin, C., Höfte, M., and Diederichsen, E. (2001) Functional Expression of *Cf9* and *Avr9* genes in *Brassica napus* induces Enhanced Resistance to *Leptosphaeria maculans*. Mol. Plant-Microbe Interact. **14**: 1075-1085.

Hobbs, S.L., Warkentin, T.D., and DeLong, C.M. (1993) Transgene copy number can be positively or negatively associated with transgene expression. PMB **21**: 171-126.

Howlett, B.J., Idnurm, A., and Pedras, M.S.C. (2001) *Leptosphaeria maculans* the causal agent of blackleg disease. Fungal Genet. Biol. **33**: 1-14.

Jakab, G., Cottier, V., Touquin, V., Rigoli, G., Zimmerli, L., Métraux, J.P., and Mauch-Mani, B. (2001) Beta-aminobutyric acid-induced resistance in plants. Eur. J. Plant Pathol. 1**07:** 29-37.

Jakab, G., Ton, J., Flors, V., Zimmerli, L., Métraux, J.P., and Mauch-Mani, B. (2005) Enhancing *Arabidopsis* salt and drought stress tolerance by chemical priming for its abscisic acid responses. Plant Physiol. **139**: 267-274.

Jefferson, R.A., Kavanagh, T.A., and Bevan, M.W. (1987) GUS fusions: beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO (Eur. Mol. Biol. Organ.) J. **6**: 3901-3907.

Jennes, A., and Kuć, J. (1979) Graft transmission of systemic resistance of cucumber to anthracnose induced by *Colletotrichum lagenarium* and tobacco mosaic virus. Phytopathol. **69**: 753-756.

Jensen, B.D., Latunde-Dada, A.O., Hudson, D., and Lucas, J.A. (1998) Protection of *Brassica* seedlings against downy mildew and damping-off by seed treatment with CGA 245704, an activator of systemic acquired resistance. Pestic. Sci. **52**: 63-69.

Jirage, D., Tootle, T.L., Reuber, T.L., Frost, L.N., Feys, B.J., Parker, J.E., Ausubel, F.M., and Glazebrook, J. (1999) *Arabidopsis thaliana PAD4* encodes a lipase-like gene that is important for salicylic acid signaling. Proc. Natl. Acad. Sci. USA. **96**: 13583-13588.

Johnson, C., Boden, E., and Arias, J. (2003) Salicylic acid and NPR1 induce the recruitment of trans-activating TGA factors to a defense gene promoter in *Arabidopsis*. Plant Cell **15**: 1846-1858.

Jones, D.A., and Jones, J.D.G. (1996) The role of leucine-rich repeat proteins in plant defences. Adv. Bot. Res. **24**: 89-167.

Jung, H.W., Kim, W., and Hwang, B.K. (2003) Three pathogen-inducible genes encoding lipid transfer protein from pepper are differentially activated by pathogens, abiotic, and environmental stresses. Plant Cell Environ. **26**: 915-928.

Kachroo, P., Yoshioka, K., Shah, J., Dooner, H., and Klessig, D.F. (2000) Resistance to turnip crinkle virus in *Arabidopsis* is regulated by two host genes and is salicylic acid dependent but *NPR1*, ethylene, and jasmonate independent. Plant Cell **12**: 677-690.

Kader, J.C. (1996) Lipid-Transfer Proteins in Plants. Annu. Rev. Plant Physiol. Plant Mol. Biol. **47**: 627-654.

Kader, J.C. (1997) Lipid-transfer proteins: A puzzling family of plant proteins. Trends Plant Sci. **2**: 66-70.

Katagiri, F., Lam, E., and Chua, N-H (1989) Two tobacco DNA-binding proteins with homology to the nuclear factor CREB. Nature **340**: 727-730.

Kauss, H. (1992) Callose and callose synthase. *In* SJ Gurr, MJ McPherson, DJ Bowles, eds, Mol. Plant Pathol. Vol. 2 Oxford University Press, Oxford, UK. pp. 1-8.

Kauss, H., Theisinger-Hinkel, E., Mindermann, R., and Conrath, U. (1992) Dichloroisonicotinic and salicylic acid, inducers of systemic acquired resistance, enhance fungal elicitor responses in parsley cells. Plant J. **2**: 655-660.

Keller, H., Blein, J-P., Bonnet, P., and Ricci, P. (1996) Physiological and molecular characteristics of elicitin-induced systemic acquired resistance in tobacco. Plant Physiol. **110**: 365-376.

Keresztessy, Z., and Hughes, M.A. (1998) Homology modeling and molecular dynamics aided analysis of ligand complexes demonstrates functional properties of lipid-transfer proteins encoded by the barley low-temperature-inducible gene family, *blt4*. Plant J. **14**: 523-533.

Kim, H.S., and Delaney, T.P. (2002) *Arabidopsis* SON1 is an F-box protein that regulates a novel induced defense response independent of both salicylic acid and systemic acquired resistance. Plant Cell **14**: 1469-1482.

Kinkema, M., Fan, W., and Dong, X. (2000) Nuclear localization of NPR1 is required for activation of *PR* gene expression. Plant Cell **12**: 2339-2350.

Klessig, D.F., and Malamy, J. (1994) The salicylic acid signal in plants. Plant Mol. Biol. **26**: 1439-1458.

Kobe, B., and Kajava, A.V. (2001) The leucine-rich repeat as a protein recognition motif. Curr. Opin. Struct. Biol. **11:** 725-732.

Kohler, A., Schwindling, S., and Conrath, U. (2002) Benzothiadiazole-induced priming for potentiated responses to pathogen infection, wounding, and infiltration of water into leaves requires the *NPR1/NIM1* gene in *Arabidopsis***.** Plant Physiol. **128**: 1046-1056.

Kuć, J. (1982) Induced immunity to plant diseases. BioScience **32**: 854-860.

Kumar, D., and Klessig, D. F. (2003) High-affinity salicylic acid-binding protein 2 is required for plant innate immunity and has salicylic acid-stimulated lipase activity. Proc. Natl. Acad. Sci. USA. **100**: 16101-16106.

Kumar, D., Gustafsson, C., and Klessig, D. F. (2006) Validation of RNAi silencing specificity using synthetic genes: salicylic acid-binding protein 2 is required for innate immunity in plants. Plant J. **45**: 863-868.

Kunkel, B.N., and Brooks, D.M. (2002) Cross talk between signaling pathways in pathogen defense. Curr. Opin. Plant Biol. **5**: 325-331.

Kus, J.V., Zaton, K., Sarkar, R., and Cameron, R.K. (2002) Age-related resistance in *Arabidopsis* is a developmentally regulated defense response to *Pseudomonas syringae*. Plant Cell **14**: 479-490.

Lamb, C.J., Lawton, M.A., Dron, M., and Dixon, R.A. (1989) Signals and transduction mechanisms for activation of plant defenses against microbial attack. Cell **56**: 215-224.

Lamb, C.J. (1994) Plant disease resistance genes in signal perception and transduction. Cell **76**: 419-422.

Lawton, K.A., Friedrich, L., Hunt, M., Weymann, K., Delaney, T., Kessmann, H., Staub, T., and Ryals, J. (1996) Benzothiadiazole induces disease resistance in *Arabidopsis* by activation of the systemic acquired resistance signal transduction pathway. Plant J. **10**: 71-82.

Lawton, K.A, Weymann, K., Friedrich, L., Vernooij, B., Uknes, S., and Ryals, J. (1995) Systemic acquired resistance in *Arabidopsis* requires salicylic acid but not ethylene. Mol. Plant-Microbe Interact. **8**: 863-870.

Lebel, E., Heifetz, P., Thorne, L., Uknes, S., Ryals, J., and Ward, E. (1998) Functional analysis of regulatory sequences controlling *PR-1* gene expression in *Arabidopsis*. Plant J. **16**: 223-233.

Lee, H-I., León, J., and Raskin, I. (1995) Biosynthesis and metabolism of salicylic acid. Proc. Natl. Acad. Sci. USA. **92**: 4076-4079.

Lenoir, A., Cournoyer, B., Warwick, S., Picard, G., and Deragon, J.M. (1997) Evolution of SINE S1 retroposons in cruciferae plant species. Mol. Biol. Evol. **14**: 934-941.

Lin, W.C., Lu, C.F., Wu, J.W., Cheng, M.L., Lin, Y.M., Yang, N.S., Black, L., Green, S.K., Wang, J.F., and Cheng, C.P. (2004) Transgenic tomato plants expressing the *Arabidopsis NPR1* gene display enhanced resistance to a spectrum of fungal and bacterial diseases. Transgenic Res. **13**: 567-581.

Liu, D., Raghothama, K.G., Hasegawa, P.M., and Bressan, R.A. (1994) Osmotin over expression in potato delays development of disease symptoms. Proc. Natl. Acad. Sci. USA. **91**: 1888-1892.

Liu, G., Holub, E.B., Alonso, J.M., Ecker, J.R., and Fobert, P.R. (2005) An *Arabidopsis NPR1*-like gene, *NPR4*, is required for disease resistance. Plant J. **41**: 304-318.

Liu, Y., Schiff, M., and Dinesh-Kumar, S.P. (2002a) Virus-induced gene silencing in tomato. Plant J. **31**: 777-786.

Liu, Y., Schiff, M., Marathe, R., and Dinesh-Kumar, S.P. (2002b) Tobacco *Rar1*, *EDS1* and *NPR1/NIM1* like genes are required for *N*-mediated resistance to tobacco mosaic virus. Plant J. **30**: 415-429.

Madamanchi, N.R., and Kuć, J. (1991) Induced systemic resistance in plants. *In* GT Cole, HC Hoch, eds, The Funga1 Spore and Disease Initiation in Plants and Animals. Plenum, New York, USA. pp. 347-362.

Madrid, S.M. (1991) The barley lipid transfer protein is targeted into the lumen of the endoplasmic reticulum. Plant Physiol. Biol. Chem. **29**: 695-703.

Mahalingam, R., Gomez-Buitrago, A., Eckardt, N., Shah, N., Guevara-Garcia, A., Day, P., Raina, R., and Fedoroff, N.V. (2003) Characterizing the stress/defense transcriptome of *Arabidopsis*. Genome Biol. **4:** R20

Mahuku, G, S., Hall, R., and Goodwin, P.H. (1996) Co-infection and induction of systemic acquired resistance by weakly and highly virulent isolates of *Leptosphaeria maculans* in oil seed rape. Physiol. Mol. Plant Pathol. **49**: 61-72.

Malamy, J., Carr, J.P., Klessig, D.F., and Raskin, I. (1990) Salicylic acid: a likely endogenous signal in the resistance response of tobacco to viral infection. Science **250**: 1002-1004.

Maldonado, A.M., Doerner, P., Dixon, R.A., Lamb, C.J., and Cameron, R.K. (2002) A putative lipid transfer protein involved in systemic resistance signalling in *Arabidopsis*. Nature **419**: 399-403.

Maleck, K., Levine, A., Eulgem, T., Morgan, A., Schmid, J., Lawton, K.A., Dangl, J.L., and Dietrich, R.A. (2000) The transcriptome of *Arabidopsis thaliana* during systemic acquired resistance. Nature Genet. **26**: 403-410.

Martin, G.B. (1999) Functional analysis of plant disease resistance genes and their downstream effectors. Curr. Opin. Plant Biol. **2**: 273-279.

Martin, G.B., Bogdanove, A.J., and Sessa, G. (2003) Understanding the functions of plant disease resistance proteins. Annu. Rev. Plant Physiol. Plant. Mol. Biol. **54**: 23-61.

Matzke, M.A., and Matzke, A.J.M. (1995) How and why do plants inactivate homologues (trans)genes? Plant Physiol. **107**: 679-685.

McDowell, J.M., and Dangl, J.L. (2000) Signal transduction in the plant immune response. Trends Biol. Sci. **25**: 79-82.

McDowell, J.M., Cuzick, A., Can, C., Beynon, J., Dangl, J.L., and Holub, E.B. (2000) Downy mildew (*Peronospora parasitica*) resistance genes in *Arabidopsis* vary in functional requirements for NDR1, EDS1, NPR1 and salicylic acid accumulation. Plant J. **22**: 523-529.

Métraux, J.-P., Signer, H., Ryals, J., Ward, E., Wyss-Benz, M.M., Gaudin, J., Raschdorf, K., Schmid, E., Blum, W., and Inverardi, B. (1990) Increase of salicylic acid at the onset of systemic acquired resistance in cucumber. Science **250**: 1004-1006.

Meyers, B.C, Kozik, A., Griego, A., Kuang, H., and Michelmore, R.W. (2003) Genome wide analysis of NBS-LRR-encoding genes in *Arabidopsis*. Plant Cell 15: 809-834.

Molina, A., and Garcia-Olmedo, F. (1997) Enhanced tolerance to bacterial pathogens caused by the transgenic expression of barley lipid transfer protein LTP2. Plant J. **12**: 669-675.

Molina, A., Segura, A., and Garcia-Olmedo, F. (1993) Lipid transfer proteins (nsLTPs) from barley and maize leaves are potent inhibitors of bacterial and fungal plant pathogens. FEBS Lett. **316**: 119-122.

Morinaga, T. (1934) Interspecific hybridization in *Brassica*. The cytology of F1 hybrids of *B. juncea* and *B. nigra*. Cytologia **6**: 62-67.

Mou, Z., Fan, W., and Dong, X. (2003) Inducers of plant systemic acquired resistance regulate NPR1 function through redox changes. Cell **113**: 935-944.

Nandi, A., Welti, R., and Shah, J. (2004) The *Arabidopsis thaliana* dihydroxyacetone phosphate reductase gene *SUPPRESSOR OF FATTY ACID DESATURASE DEFICIENCY1* is required for glycerolipid metabolism and for the activation of systemic acquired resistance. Plant Cell **16**: 465-477.

Nawrath, C., and Métraux, J.P. (1999) Salicylic acid induction-deficient mutants of *Arabidopsis* express *PR-2* and *PR-5* and accumulate high levels of camalexin after pathogen inoculation. Plant Cell **11**: 1393-1404.

Newman, M., Conrads-Strauch, J., Scofield, G., Daniels, M.J., and Dow, J.M. (1994) Defense-related gene induction in *Brassica campestris* in response to defined mutants of *Xanthomonas campestris* with altered pathogenicity. Mol. Plant-Microbe Interact. **7**: 553-563.

Osbourn, A.E. (2003) Saponins in cereals. Phytochemistry **62**: 1-4.

Osbourn, A.E., Qi, X., Townsend, B., and Qin, B. (2003) Dissecting plant secondary metabolism – constitutive chemical defenses in cereals. New Phytologist **159**: 101-108.

Ozeretskovskaya, O.L. (1995) Induced resistance in the solanaceae. In: Hammerschmidt R, Kuć J, eds. Induced Resistance to Disease in Plants. Dordrecht, Kluwer Academic, The Netherlands. pp. 31-62.

Pallas, J.A., Paiva, N.L., Lamb, C., and Dixon, R.A. (1996) Tobacco plants epigenetically suppressed in phenylalanine ammonia-lyase expression do not develop systemic acquired resistance in response to infection by tobacco mosaic virus. Plant J. **10**: 281-293.

Palva, T.K., Hurtig, M., Saindrenan, P., and Palva, E.T. (1994) Salicylic acid induced resistance to *Erwinia carotovora* subsp. *carotovora* in tobacco. Mol. Plant-Microbe Interact. **7**: 356-363.

Pan, Y., Pylatuik, J.D., Ouyang, J., Famili, A.F., and Fobert, P.R. (2004) Discovery of functional genes for systemic acquired resistance in *Arabidopsis thaliana* through integrated data mining. J. Bioinform. Comput. Biol. **2**: 639-655.

Park, S.Y., and Lord, E.M. (2003) Expression studies of SCA in lily (and confirmation of its role in pollen tube adhesion. Plant Mol. Biol. **51**: 183-189.

Parker, J.E., Holub, E.B., Frost, L.N., Falk, A., Gunn, N.D., and Daniels, M.J. (1996) Characterization of *eds1*, a mutation in *Arabidopsis* suppressing resistance to *Peronospora parasitica* specified by several different *RPP* genes. Plant Cell **8**: 2033-2046.

Parkin, I.A., Gulden, S.M., Sharpe, A.G., Lukens, L., Trick, M., Osborn, T.C., and Lydiate, D.J. (2005) Segmental structure of the *Brassica napus* genome based on comparative analysis with *Arabidopsis thaliana*. Genetics **171**: 765-781.

Pieterse, C.M.J., Van Wees, S.C.M., Hoffland, E., Van Pelt, J.A., and Van Loon, L.C. (1996) Systemic resistance in *Arabidopsis* induced by biocontrol bacteria is independent of salicylic acid accumulation and pathogenesis-related gene expression. Plant Cell **8**: 1225-1237.

Pieterse, C.M.J., Van Wees, S.C., Van Pelt, J.A., Knoester, M., Laan, R., Gerrits, H., Weisbeek, P.J., and Van Loon, L.C. (1998) A novel signaling pathway controlling induced systemic resistance in *Arabidopsis*. Plant Cell **10**: 1571-1580.

Polowick, P.L., Quandt, J., and Mahon, J.D. (2000) The ability of pea transformation technology to transfer genes into peas adapted to western Canadian growing conditions. Plant Sci. **153**: 161-170.

Prakash, S. (1980) Cruciferous oilseeds in India. In: S. Tsunoda, K. Hinata, and C. Gomez-Campo (eds.), *Brassica* crops and wild allies. Biology and breeding. Japan Scient. Soc. Press, Tokyo. pp. 151-163.

Pyee, J., Yu, H., and Kolattukudy, P.E. (1994) Identification of a lipid transfer protein as the major protein in the surface wax of broccoli (*Brassica oleracea*) leaves. Arch. Biochem. Biophys. **311**: 460-468.

Pylatuik, J.D., and Fobert, P.R. (2005) Comparison of transcript profiling on *Arabidopsis* microarray platform technologies. Plant Mol. Biol. **58**: 609-624.

Rairdan, G.J., and Delaney, T.P. (2002) Role of salicylic acid and NIM1/NPR1 in race specific resistance in Arabidopsis. Genetics **161**:803-811.

Rasmussen, J.B., Hammerschmidt, R., and Zook, M.N. (1991) Systemic induction of salicylic acid accumulation in cucumber after inoculation with *Pseudomonas syringae* pv. *syringae*. Plant Physiol. **97**: 1342-1347.

Reuveni, M., Tuzun, S., Cole, J.S., Siegel, M.R., and Kuć, J. (1986) The effects of plant-age and leaf position on the susceptibility of tobacco to blue mold caused by *Peronospora tabacina*. Phytopathol. **76**: 455-458.

Ribnicky, D.M., Shulaev, V., and Raskin, I. (1998) Intermediates of salicylic acid biosynthesis in tobacco. Plant Physiol. **118**: 565-572.

Richter, T.E., and Ronald, P.E. (2000) The evolution of disease resistance genes. Plant Mol. Biol. **42**: 195-205.

Rogers, E.E., and Ausubel, F.M. (1997) *Arabidopsis* enhanced disease susceptibility mutants exhibit enhanced susceptibility to several bacterial pathogens and alterations in *PR-1* gene expression. Plant Cell **9**: 305-316.

Ross, A.F. (1961) Systemic acquired resistance induced by localized virus infections in plants. Virology **14**: 340-358.

Rustérucci, C., Aviv, D.H., Holt, B.F. 3rd., Dangl, J.L., and Parker, J.E. (2001) The disease resistance signaling components EDS1 and PAD4 are essential regulators of the cell death pathway controlled by LSD1 in *Arabidopsis*. Plant Cell **13**: 2211-2224.

Ryals, J., Ward, E., and Métraux, J.-P. (1992) Systemic acquired resistance: An inducible defense mechanism in plants. In lnducible Plant Proteins: Their biochemistry and molecular biology, J.L. Wray, (ed) Cambridge University Press, Cambridge, UK. pp. 205-229.

Ryals, J., Neuenschwander, U.H., Willits, M.G., Molina, A., Steiner, H.Y., and Hunt, M. (1996) Systemic acquired resistance. Plant Cell **8**: 1809-1819.

Ryals, J., Weymann, K., Lawton, K., Friedrich, L., Ellis, D., Steiner, H.Y., Johnson, J., Delaney, T.P., Jesse, T., Vos, P., and Uknes, S. (1997) The *Arabidopsis* NIM1 protein shows homology to the mammalian transcription factor inhibitor I kappa B. Plant Cell **9**: 425-439.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual 2nd edn. New York: Cold Spring Harbor Laboratory Press.

Scheffler, J.A., Sharpe, A.G., Schmidt, H., Sperling, P., Parkin, I. A. P., Lydiate, D.J., and Heinz, E. (1997) Desaturase multigene families of *Brassica napus* arose through genome duplication. Theor. Appl. Genet. **94**: 583-591.

Schornack, S., Meyer, A., Romer, P., Jordan, T., and Lahaye, T. (2006) Gene-for-gene mediated recognition of nuclear-targeted AvrBs3-like bacterial effector proteins. J. Plant Physiol. **163**: 256-272.

Sedgwick, S.G., and Smerdon, S.J. (1999) The ankyrin repeat: A diversity of interactions on a common structural frame. Trends Biochem. Sci. **24**: 311-316.

Shah, J. (2003) The salicylic acid loop in plant defense. Curr. Opin. Plant Biol. **6**: 365-371.

Shah, J. (2005) Lipids, lipases and lipid modifying enzymes in plant disease resistance. Annu. Rev. Phytopathol. **43**: 229-260.

Shah, J., Tsui, F., and Klessig, D.F. (1997) Characterization of a salicylic acid-insensitive mutant (*sai1*) of *Arabidopsis thaliana*, identified in a selective screen utilizing the SA- inducible expression of the *tms2* gene. Mol. Plant-Microbe Interact. **10**: 69-78.

Shirasu, K., Nakajima, H., Rajasekhar, V. K., Dixon, R.A., and Lamb, C. (1997) Salicylic acid potentiates a gain-control amplifying pathogen signals for activation of plant disease resistance mechanisms. Plant Cell **9**: 261-270.

Shulaev, V., León, J., and Raskin, I. (1995) Is salicylic acid a translocated signal of systemic acquired resistance in tobacco? Plant Cell **7**: 1691-1701.

Siegrist, J., Jeblick, W., and Kauss, H. (1994) Defense responses in infected and elicited cucumber (*Cucumis sativus* L.) hypocotyl segments exhibiting acquired resistance. Plant Physiol. **105**: 1365-1374.

Smith, J.A., Hammerschmidt, R., and Fulbright, D.W. (1991) Rapid induction of systemic resistance in cucumber by *Pseudomonas syringae* pv. *syringae*. Physiol. Mol. Plant Pathol. **38**: 223-235.

Soufleri, I.A., Vergnolle, C., Miginiac, E., and Kader, J.C. (1996) Germination-specific lipid transfer protein cDNAs in *Brassica napus* L. Planta **199**: 229-237.

Staskawicz, B.J., Ausubel, F.M., Baker, B.J., Ellis, J.G., and Jones, J.D.G. (1995) Molecular genetics of plant disease resistance. Science **268**: 661-667.

Sterk, P., Booij, H., Schellekens, G.A., Van Kammen, A., and De Vries, S.C. (1991) Cell-specific expression of the carrot EP2 lipid transfer protein gene. Plant Cell **3**: 907-921.

Sticher, L., Mauch-Mani, B., and Métraux, J.P. (1997) Systemic acquired resistance. Annu. Rev. Phytopathol. **35**: 235-270.

Strange, R.N., and Scott, P.R. (2005) Plant disease: a threat to global food security. Annu. Rev. Phytopathol. **43**: 83-116.

Strompen, G., Gruner, R., and Pfitzner, U. (1998) An *as-1*-like motif controls the level of expression of the gene for the pathogenesis-related protein 1a from tobacco. Plant Mol. Biol. **37**: 871-883.

Subramaniam, R., Desveaux, D., Spickler, C., Michnick, S.W., and Brisson, N. (2001) Direct visualization of protein interactions in plant cells. Nature Biotechnol. **19**: 769-772.

Sutton, D.C. (1982) Field protection of bean against *Colletotrichum lindemuthianum* by *Colletotrichum lindemuthianum*. Austr. Plant Pathol. **11**: 50-51.

Tameling, W.I., Elzinga, S.D., Darmin, P.S., Vossen, J.H., Takken, F.L., Haring, M.A., and Cornelissen, B.J. (2002) The tomato *R* gene products I-2 and MI-1 are functional ATP binding proteins with ATPase activity. Plant Cell **14**: 2929-2939.

Terras, F.R.G., Godris, IJ., Van Leuven, F., Vanderleyden, J., Cammue, B.P., and Broekaert, W.F. (1992) In vitro antifungal activity of a radish (*Raphanus sativus* L.) seed protein homologous to nonspecific lipid transfer proteins. Plant Physiol. **100**: 1055-1058.

Thoma, S., Kaneko, Y., and Somerville, C. (1993) A non-specific lipid transfer protein from *Arabidopsis thaliana* is a cell wall protein. Plant J. **3**: 427-436.

Thomma, B.P.H.J., Eggermont, K., Penninckx, I.A.M.A., Mauch-Mani, B., Vogelsang, R., Cammue, B.P.A., and Broekaert, W.F. (1998) Separate jasmonate-dependent and salicylate-dependent defense-response pathways in *Arabidopsis* are essential for resistance to distinct microbial pathogens. Proc. Natl. Acad. Sci. USA. **95**: 15107-15111.

Thomma, B.P., Penninckx, I.A., Broekaert, W.F., and Cammue, B.P. (2001) The complexity of disease signaling in *Arabidopsis*. Curr. Opin. Immunol. **13**: 63-68.

Tewari, J.P., and Mithen, R.F. (1999) Diseases. In: Biology of *Brassica* Coenospecies. (ed.) C. Gomez-Campo. Elsevier Amsterdam. pp. 309-411.

Till, B.J., Reynolds, S.H., Greene, E.A., Codomo, C.A., Enns, L.C., Johnson, J.E., Burtner, C., Odden, A.R., Young, K., Taylor, N., Henikoff, J.G., Comai, I., and Henikoff, S. (2003) Large-scale discovery of induced point mutations with high-throughput TILLING. Genome Research **13**: 524-530.

Ton, J., and Mauch-Mani, B. (2004) Beta-amino butyric acid induced resistance against necrotrophic pathogens is based on ABA-dependent priming for callose. Plant J. **38**: 119-130.

Ton, J., De Vos, M., Robben, C., Buchala, A., Métraux, J.P., Van Loon, L.C., and Pieterse, C.M.J. (2002) Characterization of *Arabidopsis* enhanced disease susceptibility mutants that are affected in systemically induced resistance. Plant J. **29**: 11-21.

Ton, J., Jakab, G., Toquin, V., Flors, V., Lavicoli, A., Maeder, M.N., Métraux, J.-P., and Mauch-Mani, B. (2005) Dissecting the β-aminobutyric acid-induced priming phenomenon in *Arabidopsis*. Plant Cell **17:** 987-999.

Tsang, E.W.T., Yang, J., Chang, Q., Nowak, G., Kolenovsky, A., McGregor, I.D., and Keller, W.A. (2003) Chlorophyll reduction in the seed of *Brassica napus* with a glutamate 1-semialdehyde aminotransferase antisense gene. Plant Mol. Biol. **51**: 191-201.

Tuzun, S., and Kuć, J. (1985) Movement of a factor in tobacco infected with *Peronospora tabacina* which systemically protects against blue mold. Physiol. Mol. Plant Pathol. **26**: 321-330.

U, N. (1935) Genome analysis in *Brassica* with special reference to the experimental formation of *Brassica napus* and peculiar mode of fertilization. Japan. J. Bot. **7**: 389-452.

Uknes, S., Mauch-Mani, B., Moyer, M., Potter, S., Williams, S., Dincher, S., Chandler, D., Slusarenko, A., Ward, E., and Ryals, J. (1992) Acquired resistance in *Arabidopsis*. Plant Cell **4**: 645-656.

Uknes, S., Winter, A., Delaney, T., Vernooij, B., Friedrich, L., Morse, A., Potter, S., Ward, E., and Ryals, J. (1993) Biological induction of systemic acquired resistance in *Arabidopsis*. Mol. Plant-Microbe Interact. **6**: 692-698.

Vanacker, H., Lu, H., Rate, D.N., and Greenberg, J.T. (2001) A role for salicylic acid and NPR1 in regulating cell growth in *Arabidopsis*. Plant J. **28**: 209-216.

Van der Biezen, E.A., and Jones, J.D.G. (1998) Plant disease resistance proteins and the gene-for-gene concept. Trends Biochem. Sci. **23**: 454-456.

Van Loon, L.C., Rep, M. and Pieterse, C.M.J. (2006) Significance of Inducible Defense-related Proteins in Infected Plants. Annu. Rev. Phytopathol. **44**: 135-162.

Van Loon, L. C., and Van Strien, E.A. (1999) The families of pathogenesis-related proteins, their activities, and comparative analysis of PR-1 type proteins. Physiol. Mol. Plant Pathol. **55**: 85-97.

Van Loon, L.C., Bakker, P.A., and Pieterse, C.M.J. (1998) Systemic resistance induced by rhizosphere bacteria. Annu. Rev. Phytopathol. **36**: 453-483.

Van Peer, R., Niemann, G.J., and Schippers, B. (1991) Induced resistance and phytoalexin accumulation in biological control of *Fusarium* wilt of carnation by *Pseudomonas* sp. strain WCS417r. Phytopathol. **91**: 728-734.

Van Wees, S.C.M., Pieterse, C.M.J., Trijssenaar, A., Van 't Westende, Y.A.M., Hartog, F., and Van Loon, L.C. (1997) Differential induction of systemic

resistance in *Arabidopsis* by biocontrol bacteria. Mol. Plant-Microbe Interact. **10**: 716-724.

Van Wees, S.C.M., Luijendijk, M., Smoorenburg, I., Van Loon, L.C. and Pieterse, C.M.J. (1999) Rhizobacteria-mediated induced systemic resistance (ISR) in *Arabidopsis* is not associated with a direct effect on known defense-genes but stimulates the expression of the jasmonate-inducible gene *Atvsp* upon challenge. Plant Mol. Biol. **41**: 537-549.

Van Wees, S.C.M., De Swart, E.A.M., Van Pelt, J.A., Van Loon, L.C., and Pieterse, C.M.J. (2000) Enhancement of induced disease resistance by simultaneous activation of salicylate- and jasmonate-dependent defense pathways in *Arabidopsis thaliana*. Proc. Natl. Acad. Sci. USA. **95**: 1933-1937.

Van Wees, S.C.M., and Glazebrook, J. (2003) Loss of non-host resistance of *Arabidopsis NahG* to *Pseudomonas syringae* pv. *phaseolicola* is due to degradation products of salicylic acid. Plant J. **33**: 733-742.

Verhagen, B.W.M., Glazebrook, J., Zhu, T., Chang, H.-S., Van Loon, L.C., and Pieterse, C.M.J. (2004) The transcriptome of rhizobacteria-induced systemic resistance in *Arabidopsis*. Mol. Plant-Microbe Interact. **17**: 895-908.

Vernooij, B., Friedrich, L., Morse, A., Reist, R., Kolditz-Jawhar, R., Ward, E., Uknes, S., Kessmann, H., and Ryals, J. (1994) Salicylic acid is not the translocated signal responsible for inducing systemic acquired resistance but is required in signal transduction. Plant Cell **6:** 959-965.

Vernooij, B., Friedrich, L., Ahl-Goy, P., Staub, T., Kessmann, H., and Ryals, J. (1995) 2, 6-Dichloroisonicotinic acid-induced resistance to pathogens does not require the accumulation of salicylic acid. Plant-Microbe Interact. **8**: 228-234.

Wang, S, Y., Wu, J.H., Ng, T.B., Ye, X.Y., and Rao, P.F. (2004) A non-specific lipid transfer protein with antifungal and antibacterial activities from the mung bean. Peptides **25**: 1235-1242.

Ward, E.R., Uknes, S.J., Williams, S.C., Dincher, S.S., Wiederhold, D.L., Alexander, D.C., Hl-Goy, P., Métraux, J.P., and Ryals, J.A. (1991) Coordinate gene activity in response to agents that induce systemic acquired resistance. Plant Cell **3**: 1085-1094.

Wei, G., Kloepper, J.W., and Tuzun, S. (1991) lnduction of systemic resistance of cucumber to *Colletotrichum orbiculare* by select strains of plant growth-promoting rhizobacteria. Phytopathol. **81**: 1508-1512.

Weigel, R.R., Bauscher, C., Pfitzner, A.J., and Pfitzner, U.M. (2001) NIMIN-1, NIMIN-2 and NIMIN-3, members of a novel family of proteins from *Arabidopsis* that interact with NPR1/NIM1, a key regulator of systemic acquired resistance in plants. Plant Mol. Biol. **46**: 143-160.

Weigel, R.R., Pfitzner, U.M., and Gatz, C. (2005) Interaction of NIMIN1 with NPR1 modulates *PR* gene expression in *Arabidopsis*. Plant Cell **17**: 1279-1291.

White, R.F. (1979) Acetylsalicylic acid (aspirin) induces resistance to tobacco mosaic virus in tobacco. Virology **99**: 410-412.

Wildermuth, M.C., Dewdney, J., Wu, G., and Ausubel, F.M. (2001) Isochorismate synthase is required to synthesize salicylic acid for plant defence. Nature **414**: 562-565.

Witte, R.S. (1989) Statistics, 3rd ed. Holt, Rinehart and Winston, Inc., The Dryden Press, New York, USA.

Wolfe, J., Hutcheon, C.J., Higgins, V.J., and Cameron, R.K. (2000) A functional gene-for-gene interaction is required for the production of an oxidative burst in response to infection with avirulent *Pseudomonas syringae* pv. *tomato* in *Arabidopsis thaliana*. Physiol. Mol. Plant Pathol. **56:** 253-261.

Wyatt, S.E., Pan, S.Q., and Kuć, J. (1991) Beta-1, 3-Glucanase, chitinase, and peroxidase-activities in tobacco tissues resistant and susceptible to blue mold as related to flowering, age and sucker development. Physiol. Mol. Plant Pathol. **39**: 433-440.

Xiao, S. Calis, O., Patrick, E.E., Zhang, Z., Charoenwattana, P., Muskett, P., Parker, J., and Turner, J.G. (2005) The atypical resistance gene, *RPW8*, recruits components of basal defence for powdery mildew resistance in *Arabidopsis*. Plant J. **42**: 95-110.

Xiang, C., Miao, Z-H., and Lam, E. (1996) Coordinated activation of *as-1*-type elements and a tobacco glutathione S-transferase gene by auxins, salicylic acid, methyl-jasmonate and hydrogen peroxide. Plant Mol. Biol. **32**: 415-426.

Yalpani, N., Leon, J., Lawton, M.A., and Raskin, I. (1993) Pathway of salicylic acid biosynthesis in healthy and virus-inoculated tobacco. Plant Physiol. **103**: 315-321.

Yalpani, N., Silverman, P., Wilson, T.M.A., Kleier, D.A., and Raskin, I. (1991) Salicylic acid is a systemic signal and an inducer of pathogenesis-related proteins in virus-infected tobacco. Plant Cell **3**: 809-818.

Yan, Z. (1990) Overview of rapeseed production and research in China. Proc. Int. Canola Conf. Potash and Phosphate Institute, Atlanta. pp. 29-35.

Ye, X.S., Pan, S.Q., and Kuć, J. (1989) Pathogenesis related proteins and systemic resistance to blue mould and tobacco mosaic virus induced by tobacco mosaic

virus, *Peronospora tabacina* and aspirin. Physiol. Mol. Plant. Pathol. **35**: 161-175.

Yu, D., Chen, C., and Chen, Z. (2001) Evidence for an important role of WRKY DNA binding proteins in the regulation of *NPR1* gene expression. Plant Cell **13**: 1527-1540.

Zachowski, A., Guerbette, F., Grosbois, M., Jolliot-Croquin, A., and Kader, J.C. (1998) Characterization of acyl-binding by a plant lipid-transfer protein. Eur. J. Biochem. **257**: 443-448.

Zhang, Y., Fan, W., Kinkema, M., Li, X., and Dong, X. (1999) Interaction of NPR1 with basic leucine zipper protein transcription factors that bind sequences required for salicylic acid induction of the *PR-1* gene. Proc. Natl. Acad. Sci. USA. **96**: 6523-6528.

Zhang, B., Foley, R.C., and Singh, K.B. (1993) Isolation and characterization of two related *Arabidopsis* ocs-element bZIP binding proteins. Plant J. **4**: 711-716.

Zhang, C., Gutsche, A.T., and Shapiro, A.D. (2004) Feedback control of the *Arabidopsis* hypersensitive response. Mol. Plant-Microbe Interact. **17**: 357-365.

Zhang, Y., Tessaro, M.J., Lassner, M., and Li, X. (2003) Knockout analysis of *Arabidopsis* transcription factors TGA2, TGA5, and TGA6 reveals their redundant and essential roles in systemic acquired resistance. Plant Cell **15**: 2647-2653.

Zhou, N., Tootle, T.L., Tsui, F., Klessig, D.F., and Glazebrook, J. (1998) *PAD4* functions upstream from salicylic acid to control defense responses in *Arabidopsis*. Plant Cell **10**: 1021-1030.

Zhou, J.M., Trifa, Y., Silva, H., Pontier, D., Lam, E., Shah, J., and Klessig, D.F. (2000) NPR1 differentially interacts with members of the TGA/OBF family of transcription factors that bind an element of the *PR-1* gene required for induction by salicylic acid. Mol. Plant-Microbe Interact. **13**: 191-202.

Zhu, Q., Maher, E.A., Masoud, S., Dixon, R.A., and Lamb, C. (1994) Enhanced protection against fungal attack by constitutive co-expression of chitinase and glucanase genes in transgenic tobacco. Biotechnol. **12**: 807-812.

Ziadi, S., Barbedette, S., Godard, J.F., Monot, C., Le Corre, D., and Silue, D. (2001) Production of pathogenesis related proteins in the cauliflower (*Brassica oleracea* var. *botrytis*) – downy mildew (*Peronospora parasitica*) pathosystem treated with acibenzolar-S-methyl. Plant Pathol. **50**: 579-586.

Zimmerli, L., Jakab, G., Métraux, J.P., and Mauch-Mani, B. (2000) Potentiation of pathogen-specific defense mechanisms in *Arabidopsis* by beta-aminobutyric acid. Proc. Natl. Acad. Sci. USA. **97**: 12920-12925.

Zimmerli, L., Métraux, J.P., and Mauch-Mani, B. (2001) β-aminobutyric acid-induced protection of *Arabidopsis* against the necrotrophic fungus *Botrytis cinerea*. Plant Physiol. **126**: 517-523.

Zipfel, C., Robatzek, S., Navarro, L., Oakeley, E.K., Jones, J.D., Felix, G., and Boller, T., (2004) Bacterial disease resistance in *Arabidopsis* through flagellin perception. Nature **428**: 764-767.